Svoluji k zapůjčení své diplomové práce ke studijním účelům a prosím, aby byla vedena přesná evidence vypůjčovatelů. Převzaté údaje je vypůjčovatel povinen řádně ocitovat.

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Příprava monoklonálních protilátek proti proteinu VP2 lidských polyomavirů

Preparation of Monoclonal Antibodies Against VP2 Protein of Human Polyomaviruses

Diplomová práce

Vedoucí závěrečné práce/Školitel: RNDr. Alena Morávková, Ph.D.

Praha, 2013

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 6.5.2013

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Abstract

Aim of this diploma thesis was to prepare two protein antigens and two monoclonal antibodies, all based on VP2 minor protein of human polyomaviruses BK virus and Merkel Cell Polyomavirus. One monoclonal antibody was being prepared against unique part of VP2 protein (N-terminal epitope, not present in VP3 protein). A cell line producing such monoclonal antibody has never been established before due to low immunogenicity of the epitope. Our approach was successful in terms of mouse immunization, however, serious problems with hybridoma line stability appeared later during the preparation process. Preparation of antibody targeted to the sequence of VP2 protein of Merkel Cell Polyomavirus was another aim of this thesis. Mouse immunization and hybridoma fusion were performed successfully. After four rounds of cloning in order to purify an established clone, nine clones were cultivated in larger scale. This cultivation probably led to diminished antibody specificity and loss of production ability in most of the hybridoma cells. One more cloning should give rise to an established clone with sufficient production. Two preparations of protein antigens were performed in two expression systems. DNA encoding C-terminally truncated protein VP2 of BK virus fused with His-tag was cloned into a vector suitable for expression in *E.coli*. This approach has been both successful and unsuccessful earlier in our laboratory depending on the length of truncation. Truncation performed in this diploma thesis, together with His-tag fusion did not prove suitable for purification from a bacterial expression system. The second antigen, VP2 protein of Merkel Cell Polyomavirus, was coexpressed with VP1 major structural protein in a baculoviral expression system. A recombinant baculovirus producing proteins VP1 and VP2 was prepared. Both proteins were expressed in the system and assembled into virus-like particles. This system was used as a source of recombinant protein in this thesis, but will find further applications in our laboratory research.

Key words: BK virus, Merkel Cell Polyomavirus, antibody, hybridoma, VP2, VLPs, His-tag, S tag

Abstrakt

Cílem této diplomové práce bylo připravit dva proteinové antigeny a dvě monoklonální protilátky, založené na minoritním proteinu VP2 lidských polyomavirů BK viru a Polyomaviru karcinomu Merkelových buněk. Proběhla příprava monoklonální protilátky proti unikátní části proteinu VP2 BK viru (N-koncový epitop, který se nenachází u proteinu VP3). Buněčná linie produkující protilátku s touto specificitou nebyla zatím nikdy ustavena kvůli nízké imunogenicitě tohoto epitopu. Náš přístup byl z hlediska imunizace myší úspěšný, ale později v průběhu přípravy nastaly vážné problémy se stabilitou hybridomové linie. Dalším cílem této práce byla příprava monoklonální protilátky cílené na sekvenci proteinu VP2 Polyomaviru karcinomu Merkelových buněk. Imunizace myší a hybridomová fúze byly provedeny úspěšně. Po čtyřech kolech klonování za účelem přípravy ustaveného klonu bylo devět klonů vybráno k rozpěstování. Rozpěstování pravděpodobně vedlo ke snížení specificity protilátky a ke ztrátě produkce u většiny hybridomů. Jednou zopakované klonování by mělo vyústit v získání ustaveného klonu s dostatečnou produkcí. Příprava dvou proteinových antigenů byla provedena ve dvou expresních systémech. DNA kódující VP2 protein BK viru zkrácený na C-konci a fúzovaný s His-tagem byla klonována do vektoru vhodného pro expresi v *E.coli*. Tento přístup byl dříve v naší laboratoři úspěšný i neúspěšný v závislosti na délce zkrácení. Zkrácení provedené v této práci spojené s fúzí s His-tagem se ukázalo jako nevhodné pro purifikaci proteinu z bakteriálního expresního systému. Druhý antigen, protein VP2 Polyomaviru karcinomu Merkelových buněk, byl exprimován společně s hlavním strukturním proteinem VP1 v bakulovirovém expresním systému. Byl připraven rekombinantní bakulovirus produkující proteiny VP1 a VP2. Oba proteiny byly v systému exprimovány a skládaly se do viru podobných částic. V této diplomové práci byl systém použit jako zdroj rekombinantního proteinu, ale v laboratorním výzkumu najde i další uplatnění.

Klíčová slova: BK virus, Polyomavirus karcinomu Merkelových buněk, protilátka, hybridom, VP2, VLPs, His-tag, S tag

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Abbreviations

Ab, mAb, PAb	antibody (monoclonal, polyclonal)
BKV	BK virus
bp	base pairs
BSA	bovine serum albumin
CBBG	coomassie brilliant blue G250
CFA	complete Freund´s adjuvant
d.p.i.	days post infection
DAPI	4,6-diaminido-2-phenylindol
ddH_2O	deionized and distilled H_2O
DMSO	dimethylsulfoxide
E. coli	*Escherichia coli*
EDTA	ethylendiamintetraacetate disodium
ER	endoplasmic reticulum
ERAD	endoplasmic-reticulum-associated protein degradation
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
GFP	green fluorescent protein
h.p.i.	hours post infection
HAU	hemagglutination units
HC	hemorrhagic cystitis
HRP	horse radish peroxidase
IF	immunofluorescence
IPTG	isopropylthio-β-galactoside
JCV	JC virus
kDa	kilodaltons
LT	large T antigen
M	marker
MCC	Merkel Cell Carcinoma
MCPyV	Merkel Cell Polyomavirus
miRNA	micro RNA
MPyV	Mouse Polyomavirus
NC	negative control
NCCR	non-coding control region

NLS	nuclear localisation signal
NSCLC	non-small cell lung carcinoma
OD	optical density
ORF	open reading frame
ori	origin of replication
PBS	phosphate-buffered saline
PC	positive control
PCR	polymerase chain reaction
PDI	protein disulphide isomerase
PH	polyhedrin
PVAN	polyomavirus-associated nephropathy
RFU	relative fluorescence units
RPTE cells	renal proximal tubular epithelial cells
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	short interfering RNA
st	small T antigen
SV40	Simian Vacuolating virus 40
TEM	transmission electron microscopy
TEMED	tetramethylethylendiamin
truncT	truncated T antigen
VLP	virus-like particle
WT	wild type

1. Introduction and Aims of the Thesis

The *Polyomaviridae* family is a viral family, the importance of which is currently rising. Several human pathogens can be found among polyomaviruses. The amount of described polyomaviruses infecting humans has multiplied during the last decade and discoveries of further ones are anticipated. The importance and danger of human polyomaviruses lies in more aspects. Very little is known about their life cycle and pathogenicity. Reported data imply their long-term and complicated roles in pathogenesis. Almost no human polyomavirus was proven to be necessary and sufficient for any known disorder. Viral impact is usually enhanced by immunosuppression and other (usually unknown) aspects before clinical symptoms are visible.

This diploma thesis is focused on two human polyomaviruses – BK virus, the first discovered human polyomavirus and Merkel Cell Polyomavirus – the first polyomavirus directly connected with human cancer. Despite the fact that BK virus was discovered more than 40 years ago, its role in human pathogenesis and especially its life cycle have not been sufficiently described till today. Since BK virus is ubiquitous in humans and its role in kidney disorders was recognized, it became a subject of intensive clinical research. This research revealed important facts about extremely high prevalence of this virus in human population and suggested influence of BK virus on various disorders including cancer. The clinical research also shows clearly, that incidence of BK viral-associated disorders is on the rise. Unfortunately, even about a pathogen as prevalent and currently rising as BK virus is, we know almost nothing about its life cycle and infection on the molecular level. This kind of information is, however, necessary, if you intent to start developing a treatment. First we need to understand the virus and its life cycle and only then we can find and target the right site to fight the infection.

In Merkel Cell Polyomavirus the situation is not quite the same, but the lack of information is equal. It is one of the most lately discovered human polyomaviruses and is directly connected with one disorder – the Merkel Cell Carcinoma (even though roles in other tumors are also suspected). Like in the case of BK virus, the reported data about Merkel Cell Polyomavirus usually originate from clinical research and very little is known about the molecular biology or infection of the virus. It is noteworthy that Merkel Cell Polyomavirus research is extremely limited by the fact that a productive infection was never achieved and infectious virions were never generated under laboratory conditions. Although the examination of life cycle by standard methods is absolutely impossible, research is conducted on single proteins *in vitro*.

Polyomaviral proteins both structural and nonstructural, as is the case for many viruses, are multifunctional and necessary during infection. Polyomaviral minor structural proteins (VP2 and VP3, present in viral capsid in 72 copies) possess functions indispensable for successful productive infection in various phases of infection. Even though these phases were roughly described (but only

for model polyomaviruses – Mouse Polyomavirus (MPyV) and Simian Vacuolating virus 40(SV40)), details including exact localization of minor proteins are insufficiently covered. Especially the meaning of encoding two minor proteins, one of which is a shorter version of the other (VP3 sequence is N-terminally truncated VP2 sequence) is a challenging question to address.

One of the most powerful tools used on a daily basis in the molecular biology research are monoclonal antibodies. The ability of antibodies to detect almost any epitope in the all-or-nothing manner together with their easy visualization and high stability makes them an indispensable equipment of every laboratory conducting research on proteins. While examining viral life cycle, it is very important to describe localization of viral proteins and also their colocalization with cellular proteins. Since monoclonal antibodies against minor proteins of BK virus and Merkel Cell Polyomavirus are not available commercially, it is inevitable to undergo the long and laborious procedure of their preparation.

The aims of the diploma thesis:

1. To prepare a monoclonal antibody against N-terminal unique part of VP2 protein of BK virus

2. To prepare a monoclonal antibody against VP2 protein of Merkel Cell Polyomavirus

3. To prepare a bacterial system for expression of C-terminally truncated VP2 protein of BK virus

4. To prepare a recombinant baculoviral system for expression of VP1 and VP2 proteins of Merkel Cell Polyomavirus

2. Literature review

2.1 Human polyomaviruses

Polyomaviruses form a *Polyomaviridae* family of non-enveloped small viruses bearing circular covalently closed dsDNA as a genome. Until 2000 they were recognized as members of the family *Papovaviridae.* This family was split into *Papillomaviridae* and *Polyomaviridae* in 2000 based on the Seventh Report of the International Committee on Taxonomy of Viruses (Kryczynski, 2000). It is thus not surprising that these two families share certain characteristics and researchers often inspire their hypothesis based on the other family's characteristics.

Polyomaviral infection is rather silent in terms of visible clinical symptoms. Majority of human polyomaviruses has not been assigned to a disease until today. That is why only modern methods of detection (based on recognition of viral nucleic acid rather than cytopathic effect or a disease) can reveal new polyomaviruses. That explains why an outburst of discoveries of new human polyomaviruses took place over last 5 years, while others are still anticipated (Feltkamp et al., 2013).

So far, 10 human polyomaviruses have been discovered. They belong to three polyomaviral lineages – *Orthopolyomavirus*, *Malawipolyomavirus* and *Wukipolyomavirus* (Fig. 2.1). In 1971, the first human polyomavirus was discovered in urine of a renal allograft recipient, using cytology and electron microscopy (Gardner et al., 1971). It was named after the patient as BK virus. At the same time, a polyomavirus was discovered in brain tissue of a patient with progressive multifocal leucoencephalopathy, using cultivation of brain extracts on tissue cultures (Padgett et al., 1971). It was named after the patient as JC virus. Both BK virus and JC virus (JCV) are very common in general population, seroprevalence reaches 82 % and 39 %, respectively (Kean et al., 2009). In 2007, another human polyomavirus was discovered in a collection of nasopharyngeal aspirates using random-PCR (Allander et al., 2007). It was named KI virus after the Karolinska Institute in Stockholm, the scene of the discovery. In 2007, one more polyomavirus was discovered in a nasopharyngeal aspirate using a shotgun sequencing strategy (random-PCR from total nucleic acid) (Gaynor et al., 2007). It was named WU virus after Washington University in St Luis, the scene of the discovery. Despite the fact that prevalence of KI and WU viruses is often low in general population, the high seroprevalence (55% and 69%, respectively) (Kean et al., 2009) suggests that infections of these viruses are rather common. In 2008 the first human polyomavirus directly connected to cancer was discovered in Merkel Cell Carcinoma cells using digital transcriptome subtraction (Feng et al., 2008) (see chapter 2.1.2. for more details). The virus was named after the carcinoma cells, Merkel Cell Polyomavirus. The seroprevalence data differ significantly among various viral isolates and various studies, ranging from 25 % - 40 % (Stolt et al., 2003) to 80 % (Pastrana et al., 2009). In 2010, three novel polyomaviruses were discovered – Trichodisplasia Spinulosa-associated virus in plucked facial spines

of a heart recipient with trichodisplasia spinulosa (van der Meijden et al., 2010), human polyomavirus 6 and human polyomavirus 7 in forehead swab samples of healthy individuals (Schowalter et al., 2010), all of them using rolling-circle amplification. In 2011, human polyomavirus 9 was discovered in human serum using PCR with degenerate polyomavirus primers (Scuda et al., 2011). The last human polyomavirus so far discovered is Malawi polyomavirus. It was found in a healthy child´s stool using pyrosequencing of isolated viral DNA (Siebrasse et al., 2012). It was named after the country of origin of the child.

As mentioned above, polyomaviral infection is usually not connected to visible symptoms or a disease. The main danger of polyomaviruses is their tumorogenic potential. Polyomaviruses have been shown to be strictly host- and tissue-specific. When a polyomavirus does infect diverse cells (regardless of its specificity), the infection often results in cancer development, as has been proven in animal models (Dalianis and Hirsch, 2013). Polyomaviruses are not only very common invaders of general population (Kean et al., 2009), they are also one of the most stable viruses. Inactivation of polyomaviruses by dry heat is inefficient, moist heat is efficient only in combination with altered pH (<4.5 or >10) and long incubation. Viral resistance to UV irradiation is also excessively high. As the tumorogenic potential is not conditioned by presence of complete genome, it is even more difficult to inactivate polyomaviral tumorogenicity than their infectivity (Nims and Plavsic, 2013).

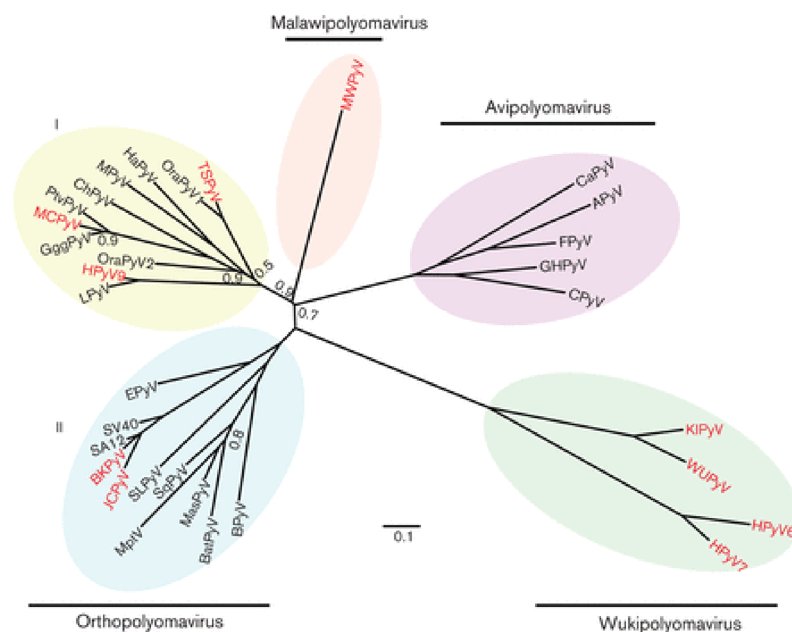


Fig. 2.1: Phylogenetic tree of polyomaviruses. Human polyomaviruses are shown in red and belong to three lineages – *Malawipolyomavirus*, *Wukipolyomavirus* and the biggest *Orthopolyomavirus*. There is a significant evolutional diversity among the members of *Orthopolyomavirus*, this fact is depicted by dividing the lineage into groups I a II. MCPyV and BKV belong to different groups of *Orthopolyomavirus*, which is in agreement with significant differences between these two viruses. (Feltkamp et al., 2013)

2.1.1. BK polyomavirus

BK polyomavirus (BKV) was discovered as the first polyomavirus infecting humans (together with JCV) in 1971. Gardner et al. (1971) examined a urine sample from a renal allograft recipient by electron microscopy and observed cytopathic effect in cell culture. They also postulated the kinship of BK virus with SV40 based on crossreactivity of sera.

As the virus was discovered before *Polyomaviridae* were approved as viral family, it was originally classified into the Polyoma subgroup of Papoviruses (Gardner et al., 1971). Currently, BK virus belongs to *Orthopolyomavirus* lineage of the *Polyomaviridae* family, together for example with JC virus or Merkel Cell Polyomavirus (see chapter 2.1.). Even though BK virus was discovered more than forty years ago and as a human pathogen it is an important research object, there are still many unanswered questions till today. The reason for this lies in various research difficulties – an animal model for BK viral infection is lacking, cell line appropriate for BK viral infection (human RPTE cells, renal proximal tubular epithelial cells (Low et al., 2004)) has been found decades after the virus discovery. Before then, research had been conducted on other cell lines, mainly Vero cells, which naturally led to result bias.

2.1.1.1. Virion structure and genome

BK virus is a typical member of the *Polyomaviridae* family, possessing all the structural properties defining polyomaviruses. It is a non-enveloped virus with an icosahedral capsid and a circular dsDNA for genome (Bennett et al., 2012).

Viral genome contains 5153 bp (Fig.2.2), harboring genes coding for 7 proteins and a non-coding control region (NCCR) situated around the origin of replication. NCCR contains not only the origin of replication, but also promoters and various regulatory factors binding sites (Cole, 2001). NCCR is a site of frequent mutations during infection *in vivo*. Virus isolates from various locations in patient's body usually differ in NCCR from the transmissible virus found in urine (so called archetype). These mutations might function in enhancing T antigen transcription as this is the case for JCV (Gosert et al., 2010).

Two coding regions are usually referred to as early and late coding regions. They are localized on different DNA strands and their expression takes place in different times post infection. The early region codes for three proteins – large tumor antigen (LT), small tumor antigen (st) and truncated tumor antigen (truncT) (Bennett et al., 2012). Open reading frames (ORF) of these proteins are the same, the final proteins differ due to alternative splicing. T antigens, or early proteins, are expressed first in the life cycle and possess impressive number of regulatory functions (see chapter 2.1.1.2.).

The late region codes for four proteins – structural proteins VP1, VP2 and VP3 and agnoprotein. ORF of VP2 and VP3 overlap, VP3 being a truncated version of VP2 (Fig.2.2). Late proteins are expressed after initiation of viral replication. Structural proteins VP1, VP2 and VP3 form the viral capsid after translocation to the nucleus (Bennett et al., 2012). Agnoprotein is one of the late proteins, however, its function is not structural.

Agnoprotein, as the name suggests (*agnosis* meaning "without knowledge" in Greek) posses many functions, some of them probably not yet described. Agnoprotein is an important regulator in a virus infection, one of the described functions is regulation of early gene expression when late genes are expressed and viruses mature. It binds various viral and cell proteins, can be phosphorylated, is found in different locations within the cell, which suggests that additional roles for agnoprotein are yet to be discovered. Virus lacking agnoprotein is viable, but with significantly reduced infectivity. Among human polyomaviruses described so far, only BKV and JCV code for agnoprotein. How can other human polyomaviruses survive without such a potent regulator is not known. One of hypotheses suggests that since polyomaviral infections are usually ubiquitous in humans, other human polyomaviruses could profit from coinfection and use BK viral agnoprotein already present in the cell (Gerits and Moens, 2012). Another regulator function encoded in the genome is held by a micro RNA (miRNA), which was shown to act in inhibition of NK T-cell activity (Bauman et al., 2011).

Viral capsid consists of the three structural proteins, major protein VP1 forms pentamers which are structural units of the capsid. Inside each VP1 pentamer, there is one copy of minor protein VP2 or VP3 attached by hydrophobic interactions to the VP1 pentamer cavity (Fig. 2.3) producing a capsomer (Sapp and Day, 2009). Minor proteins VP2 and VP3 are not in an equal stoichiometric ratio (Bauman et al., 2011) and they are completely hidden in the intact mature capsid, which complicates their research significantly (see chapter 2.1.3.). Capsomers, building units of the capsid, interact by sequence elements inserting into neighboring capsomers and by disulphide bonds, 72 capsomers form the viral capsid (Sapp and Day, 2009). BK viral capsid is an icosahedrons in shape (icosahedral lattice T = 7), 40 - 45 nm in diameter. The capsid protects viral minichromosome – circular DNA genome with cellular histones (except for H1) (Gerits and Moens, 2012). The capsid is not enveloped with a lipid bilayer and it is extraordinarily stable under various conditions (Nims and Plavsic, 2013) as described above.

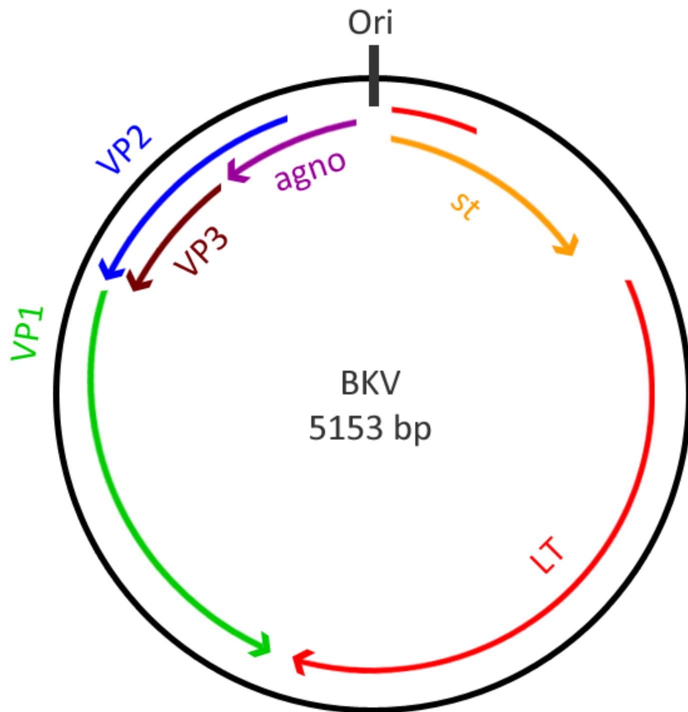


Fig.2.2: Scheme of BK viral genome, origin of replication and viral genes are represented. NCCR is localized around the origin of replication (ori). Semicircle on the right represents the early region coding for LT and st (truncTAg is not shown). All the T antigens are transcribed from the same ORF and undergo alternative splicing. Semicircle on the left represents late region coding for VP1, VP2, VP3 and agnoprotein. ORF of VP2 and VP3 overlaps, VP3 being a shorter version of VP2.

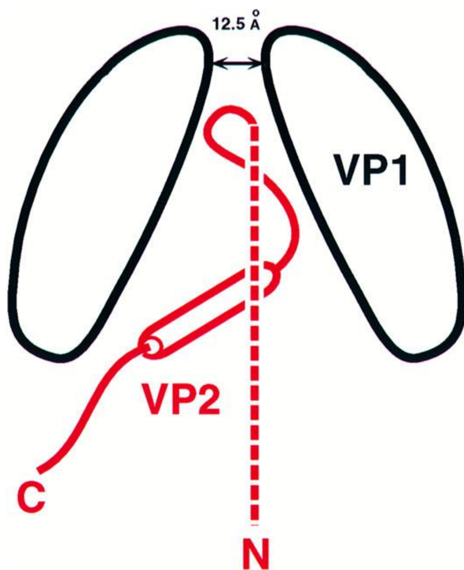


Fig.2.3: Cross-section of a capsomer, a building unit of polyomaviral capsid. Minor protein VP2 (VP3) is shown to be inserted into a pentamer of VP1 proteins, bound by hydrophobic interactions. Dashed line represents N-terminal unique part of VP2 protein (not present in VP3) (Chen et al., 1998)

2.1.1.2. Life cycle

Not much is known about BK viral life cycle, but many aspects can be adopted from a close relative – Simian Vacuolating virus 40 (SV40), which was studied thoroughly as an important model virus. BK viral infection begins by attachment to receptor, BK virus uses gangliosides GD1b and GT1b, both sharing a α2-8-linked disialic acid motif (Low et al., 2006). The affinity to receptor is rather low, which suggests that the binding is maintained by repetitive viral ligands (Sapp and Day, 2009). The crystal structure of virus-receptor interaction was not solved and the exact binding site is not known. For BK virus infection *in vitro* the renal proximal tubular epithelial cells (RPTE cells) were recognized as a relevant cell culture (as described above), but BKV was also shown to enter cell lines derived from breast cancer or melanomas, but with low efficiency (Schowalter et al., 2012).

After receptor binding and internalization into the cell, BK virus uses small non-coated non-clathrin vesicles to traffic through cytoplasm. These vesicles were defined as caveosomes (Dugan et al., 2006), but lately caveosomes were labeled as non-existing research artifacts (Parton and Howes, 2010) and so the vesicle type needs reconsideration. BK viral pathway through cytoplasm finishes in endoplasmic reticulum (ER), but individual steps are still misty, some hypotheses have been adopted based on knowledge about SV40. In terms of cytoskeleton, BK virus seems to be dependent on microtubules and actin-independent. Acidification is required for BKV early after internalization, which suggests that the trafficking probably leads through endosome compartments as was shown for SV40 (Bennett et al., 2012).

BKV was shown to traffic through endoplasmic reticulum (Sapp and Day, 2009) where it undergoes necessary conformational changes in cooperation with ER-resident proteins like protein disulphide isomerase (PDI) ERp29 or Derlins (Jiang et al., 2009). As was shown for SV40, these conformational changes result in exposition of minor proteins VP2 and VP3 in ER (Kuksin and Norkin, 2012). Even though it is not yet clear whether the whole polyomaviral virion or just genome exits ER (Sapp and Day, 2009), minor proteins play important roles in this step. Minor proteins of mouse polyomavirus (MPyV) were shown to bind to and perforate membrane of endoplasmic reticulum (Rainey-Barger et al., 2007), viruses lacking these proteins have severe problems already during early steps of infection (Mannová et al., 2002), which points to a role connected to ER-cytoplasm trafficking. As shown for SV40, nuclear entry of the partly disassembled virion is maintained by importin α2/β heterodimer. The importin heterodimer binds to nuclear localization signal (NLS) of VP3 protein (Nakanishi et al., 2002) and this binding is both necessary and sufficient for genome nuclear entry (Nakanishi et al., 2007). Similar description of BK virus or other polyomaviruses trafficking is not available.

After nuclear entry, expression of early genes starts immediately. When sufficient amount of T antigens is produced to ensure appropriate conditions – unwinding viral genome for transcription, inhibiting tumor suppressors p53 and pRb and enhancing cell cycle progression to S phase - viral replication begins. As the cell is forced to enter S phase by LT, sufficient amount of DNA polymerase and additional factors is present to be used by viral machinery. BK viral LT binds to origin of replication (ori) on viral chromosome and unwinds the DNA, it also brings cellular DNA polymerase to ori and the replication begins (Bennett et al., 2012). Right afterwards, late genes expression is started producing structural proteins and agnoprotein which regulates early gene expression (Gerits and Moens, 2012). Each of the three structural proteins contains NLS, which leads them to the nucleus where virion assembly occurs (Bennett et al., 2012). BK viral assembly is not well described, but again some results found for SV40 can come handy to make a rough picture. Novel capsids are formed around the viral minichromosome in the nucleus. Histones, VP1, VP2 and VP3 all posses DNA binding domains, the interactions are not perfectly understood nowadays, but for SV40 VP2 and VP3 a role similar to histone H2A in compacting viral minichromosome was proposed (Clever et al., 1993). VP2 and VP3 probably posses more functions during virion assembly, as they are necessary for SV40 for *in vitro* assembly of VP1 into capsids at physiological pH (Kawano et al., 2006). Once virions are assembled, cell lysis occurs probably as simple consequence of virus accumulation (Drachenberg et al., 2003) and the progeny is released.

2.1.1.3. Pathogenicity

BK virus as other polyomaviruses is highly host- and tissue-specific (Dalianis and Hirsch, 2013). A respiratory route was proposed as the main route of transmission for BK virus based on both epidemiological data and presence of BKV in respiratory tract. In human body, BK virus settles a life-long persistent infection in kidneys and urinary tract (Abend et al., 2009). Character of the persistent infection was not completely described, so it is unclear whether virus enters a true latency or rather keeps genome expression low (Bennett et al., 2012).

The persistent infection is mostly asymptomatic, it is sometimes accompanied by virus shedding in urine (Girmanova et al., 2012; Kawano et al., 2006) or saliva (Robaina et al., 2013) in healthy individuals. BKV infection reactivates under immunosupression (Feltkamp et al., 2013), various diseases were shown to occur consequently to the reactivation (Bennett et al., 2012), but two of them predominate – hemorrhagic cystitis (HC) characterized by viral antigen induced acute inflammation and polyomavirus-associated nephropathy (PVAN) characterized by inflammation as a consequence of cytolysis caused by viral replication (Dalianis and Hirsch, 2013). Both HC and PVAN occur predominantly following kidney transplantation since it is accompanied by immunosuppressive

treatment. This was the case in the discovery of BK virus in 1971 as well, kidney allograft donor and recipient were both infected with BK virus prior to transplantation and BKV was discovered in high titers in urine (Gardner et al., 1971). BKV associated diseases are on increase lately due to the development of more potent immunosupressives (Allander et al., 2007). The forfeit for decreased graft rejections under immunosupression is paid by increased BKV reactivation.

In terms of HC and PVAN treatment, reduction of immunosupression is often efficient. Antiviral drugs targeted on BKV have not been developed so far, but some approved inhibitors of DNA polymerases like Cidofovir, CMX001, Leflunomide and others are used in patients' treatment (Gardner et al., 1971). Other aspects seem to be important for BKV treatment, for example the type of dialysis procedure after renal replacement treatment was found to significantly influence BKV replication (Mitterhofer et al., 2012).

In terms of immune reaction, BK viral persistence is accompanied by cytokine expression and B and T cell proliferation. In short-term response, mainly antiapoptotic pathways are activated. In long-term response, T cell cytotoxicity becomes prevalent (Girmanova et al., 2012). MicroRNA targeting on NK cells is encoded in the viral genome, which indicates the importance of NK cells in the immune response. Generally, strong response of cytotoxic T lymphocytes and weak antibody response are associated with viral clearance (Allander et al., 2007) as well as low cytokine production (Low et al., 2006). This is in agreement with high titers of antibodies in patients with high viral loads and low efficiency of using neutralizing immunoglobulins for treatment (Gardner et al., 1971).

Another important aspect of BK viral presence in human body is its tumorogenic potential. Even though BK viral function in tumorogenesis has not been proven so far (Abend et al., 2009), as a polyomavirus it definitely possesses the potential to transform cells. As mentioned above, polyomaviruses are strictly host-and tissue-specific. In the rare cases when polyomavirus appears in different host, infection is abortive. During the abortive infection, LT impact is isolated from viral life cycle, which often results in malignant transformation (Dalianis and Hirsch, 2013). BK virus was shown to cause malignant transformation in animal newborns or to transform cell cultures *in vitro*. Cells infected with BK virus become so called “decoy cells” - typical cells with nuclear enlargement and viral inclusions. These "decoy cells" belong to risk factors for bladder cancer when present in urine (Abend et al., 2009). BKV presence in primary tumor and metastases, but not in surrounding tissue was described (van Aalderen et al., 2013). Also a case of complete resolution of renal cell carcinoma metastases after removal of immunosupresives was described (Neirynck et al., 2012). A connection between BKV and prostate cancer as well as adrenal cancer was proposed. On the other hand, results related to viral presence in tissues are somewhat disagreeing even when the same

samples are analyzed by different laboratories. The detection is also complicated by the fact that BKV infection is ubiquitous in healthy individuals and it is often difficult to distinguish BKV presence in tumor cells from its presence in normal tissue (Abend et al., 2009). To complicate the situation even more, general impact of viruses on cancer is not resolved either. Viruses often seem to contribute significantly to cancer development, but their role can take place considerably long before the appearance of tumor (Moore and Chang, 2010). Finding evidence for BKV role in cancer development is a question yet to be addressed. So far it seems that BKV contributes to cancer development, but it is probably not sufficient by itself (Abend et al., 2009).

2.1.1.4. Prevalence

BK virus is ubiquitous in humans (Bennett et al., 2012). Antibodies against BKV antigens can be found in majority of population – reaching usually more than 80% (VP1-based ELISA detection found 82% sera to be positive (Kean et al., 2009)). In terms of seroprevalence it is the most frequent one of human polyomaviruses discovered so far. The seroprevalence is not gender related, but it seems to be age related (which is unique among human polyomaviruses) – most children develop antibodies before reaching the age of ten, but after the age of fifty specific antibodies start to wane (Kean et al., 2009).

Seroprevalence is very high as mentioned above, but in virus prevalence itself, the case is markedly different (Feltkamp et al., 2013) . Even though BKV DNA can be found in healthy individuals in urine (Girmanova et al., 2012; Kawano et al., 2006) or saliva (Robaina et al., 2013), the prevalence in population reaches only 5 – 10 %. This prevalence reaches about 25 % in pregnant women (Csoma et al., 2012) probably as a result of immunosuppression and following virus reactivation. The epidemiological data in agreement with evidence from the life cycle and infection research (as mentioned above) suggest that BK viral infection happens early in childhood and then virus settles persistent nonproductive infection, invisible in terms of antigen production. Since BKV specific antibodies are found very early in children (Kean et al., 2009), there is a suspicion on vertical transmission. BK virus was found in aborted fetuses and their placentas as well as in placentas from normal childbirths. Furthermore, when viral isolates from mothers and placentas/fetuses were compared, they showed high homology in regulatory regions (NCCR, the hotspot for mutations as mentioned above). The authors thus propose a hypothesis that vertical transmission is a route of transmission for BK virus, and they suggest it could be the dominant route (Pietropaolo et al., 1998).

2.1.2. Merkel Cell Polyomavirus

Merkel Cell Polyomavirus (MCPyV) is the first human polyomavirus directly linked to cancer, the Merkel Cell Carcinoma (MCC). It was discovered while searching for the cause of Merkel Cell Carcinoma. There was a suspicion that the cause of MCC was a pathogen since incidence increases during immunosuppression. Feng et al. (2007) developed a method called digital transcriptome subtraction and used it to screen transcriptomes of MCC samples (Feng et al., 2008). They discovered a polyomavirus closely related to Lymphotropic Polyomavirus and named it based on the disease as Merkel Cell Polyomavirus. They also described that MCPyV was integrated randomly in genome, was found in 80 % of MCC and confirmed that virus isolates from the carcinoma and derived metastases were identical.

MCPyV belongs to the *Orthopolyomavirus* lineage of the *Polyomaviridae* family, together with for example BKV, JCV or SV40 (Feltkamp et al., 2013). MCPyV quickly became a hot topic for research even though the research is limited by various complications. Cells permissive for infection have not been found until today, although some clues indicate they are localized in skin (Schowalter et al., 2012) and it is probable that Merkel cells are not permissive (Tilling and Moll, 2012). Cell lines derived from various tumors proved to be almost completely non-permissive with highly restricted replication (Schowalter et al., 2012). Only cell lines with additional expression of polyomaviral LT (293TT) are successfully transduced by MCPyV genome, although not by all MCPyV isolates (Pastrana et al., 2009). The most successful attempt to perform a productive infection was made by Schowalter et al. (2011), who established a cell line named 293-4T expressing MCPyV LT and st antigens. In this cell line, they were able to propagate new virions, although in modest levels. Many authors suggest a parallel between MCPyV and high risk human papillomaviruses ((Feng et al., 2008) and others). Papillomaviral life cycle is highly dependent on differentiation of keratinocytes and it took many years to figure out details of the whole process (Sapp and Day, 2009). Regardless the predicted complexity of life cycle and the limitations described above, MCPyV is an intensively investigated virus. Despite the intensive research, MCPyV productive infection hasn't been achieved so far and description of infectious virus is yet to be reported.

2.1.2.1. Virion structure and genome

Since the MCPyV productive infection was never performed at laboratory conditions, little is known about the structure of the capsid. But based on reported data regarding the genome (Spurgeon and Lambert, 2013) or on observations of virions shed from human skin (Schowalter et al., 2010), MCPyV is a typical member of the *Polyomaviridae* family. This means that its virion is an icosahedron in shape (icosahedral lattice T = 7), composed of 72 pentamers of protein VP1 and stabilized by Ca^{2+}

ions (Sapp and Day, 2009). Each pentamer harbors one minor structural protein, VP2 or VP3 (Fig. 2.3), although there is a debate about the length and also the functionality and the very existence of MCPyV VP3 protein (Spurgeon and Lambert, 2013), since a genetic region coding for VP3 present in other polyomaviruses is lacking in MCPyV genome (Pastrana et al., 2009).

Genome consists of early region, late region and NCCR (Fig.2.4). The NCCR harbors origin of replication and binding sites for regulation factors (Spurgeon and Lambert, 2013). Unlike the NCCR in BK virus, NCCR in MCPyV is rather invariable, sequence variability in NCCR sequences among viral isolates is low (Schowalter et al., 2010). The whole genome is rather stable and differs only in point mutations among various isolates, as shown by clinical data (Martel-Jantin et al., 2012). The early region encodes large (LT) and small (st) T antigens whose role lies in regulation and 57 kT antigen, which is probably an analog of SV40 17kT antigen, but its function has not been sufficiently described so far. The three T antigens are transcribed from the same ORF and undergo alternative splicing, resulting in presence of different domains (Fig.2.5). Even though this setting of the early region is conserved among polyomaviruses, MCPyV LT shares only about 30% homology with LT of other human polyomaviruses (Johnson, 2010). The late region encodes structural proteins VP1, VP2 and a predicted VP3 (the length and existence of which is doubted as described above). The switch between early and late genes expression is probably mediated by accumulation of T antigens and following virus replication. Certain regulatory functions are possessed by miRNAs encoded in the genome (Fig.2.6) (Lee et al., 2011). MCV-miR-M1-5P was found to be expressed in low levels in 50 % of Merkel Cell Carcinomas and based on its complementarity to LT, its role was proposed to be in LT regulation during late phases of infection (Lee et al., 2011). MCV-miR-M1-3P was also proposed to function in regulation of early transcripts (Seo et al., 2009), as this is the case for miRNAs described for other polyomaviruses, for example for BKV (see chapter 2.1.1.1.).

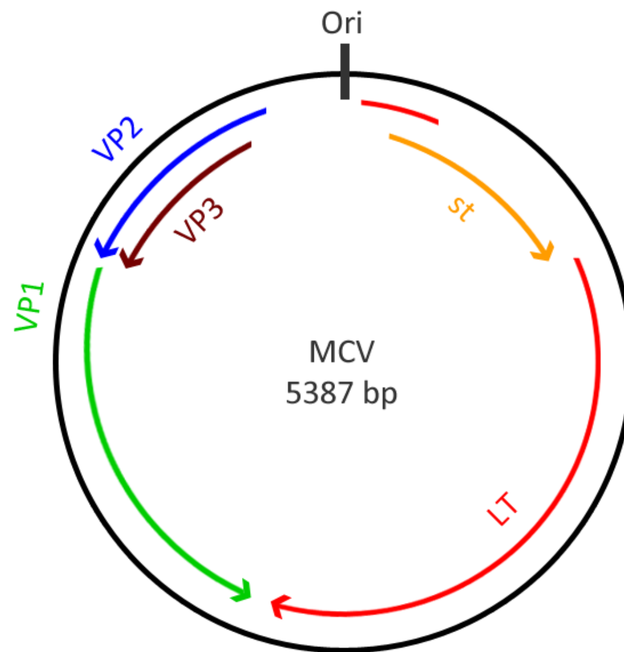


Fig.2.4: MCPyV genome scheme. NCCR region is localized around the origin of replication (ori). Semicircle on the right represents the early region coding for LT and st, 57 kT is not shown. All three T antigens share a single ORF and undergo alternative splicing. Semicircle on the left represents the late region coding for VP1, VP2 and (predicted) VP3. Other genes present in polyomaviruses like those coding for agnoprotein or middle T antigen are lacking in MCPyV genome.

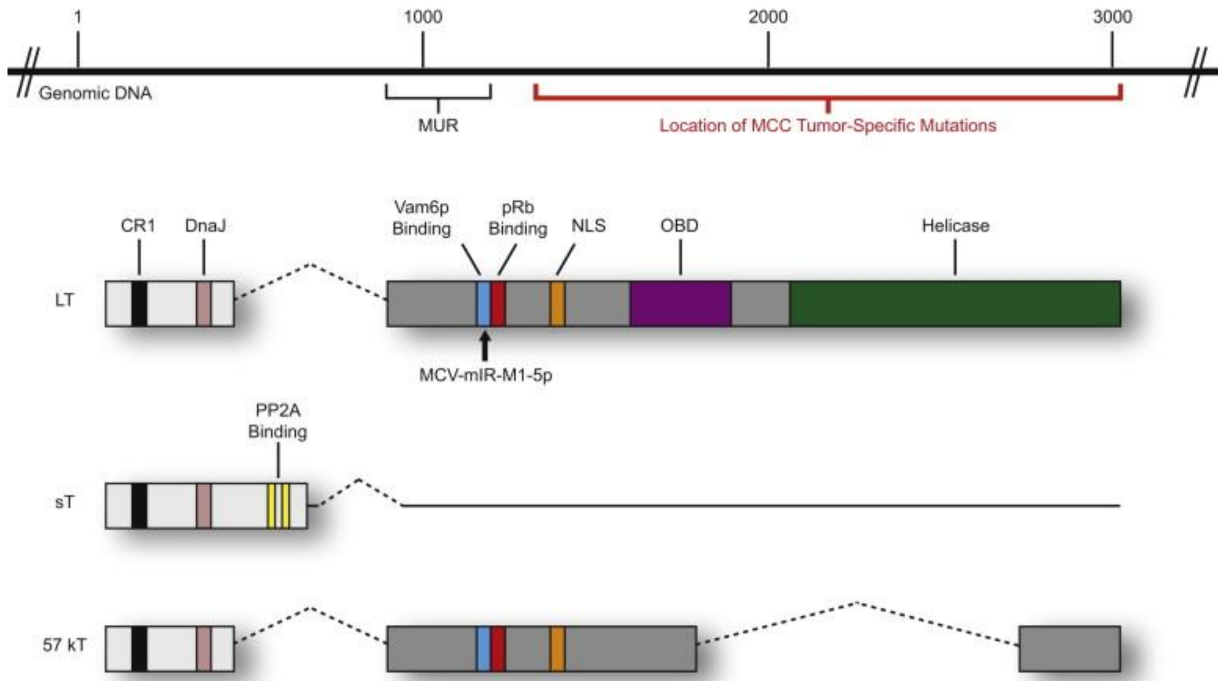


Fig.2.5 : The MCPyV T antigens ORF and domains represented in each T antigen following alternative splicing. Conserved region 1 (CR1, black) is conserved in all T antigens together with DnaJ domain (pink) which binds to heat shock proteins. A domain binding PP2A phosphatase (PP2A binding, yellow) is unique for st antigen. LT and 57kT both include nuclear localization signal (NLS, orange) and domains binding proteins Vam6 (Vam6p binding, blue) and pRb (pRb binding, red). In addition to above mentioned domains, LT includes an origin-binding domain (OBD, purple) and helicase domain (Helicase, green) which is indispensable in virus replication. (Spurgeon and Lambert, 2013).

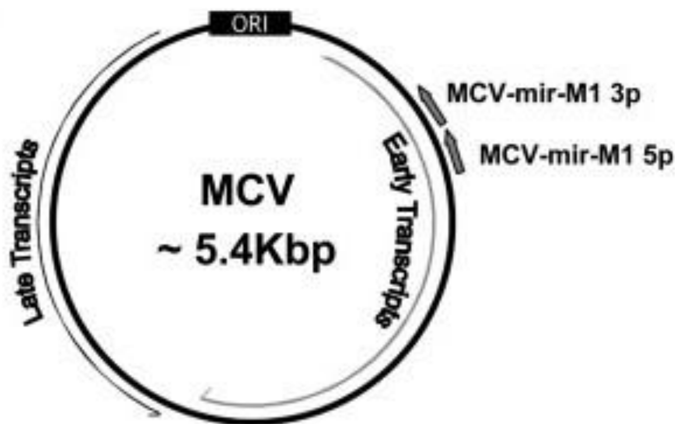


Fig.2.6: MCPyV genome with localization of miRNAs MCV-miR-M1-3P and MCV-miR-M1-5P, possessing reverse homology to early gene region and functioning probably in regulation of early gene transcription during late times post infection (adapted from (Seo et al., 2009)).

2.1.2.2. Life cycle

Polyomaviral life cycle, especially the trafficking to nucleus, is a very complex process. It has not been satisfactorily described even for polyomaviruses that have been already studied for decades. Taken into consideration that no laboratory system for successful MCPyV propagation has been established, it is not surprising that the data about MCPyV life cycle are very scarce.

Some obtained data indicate rather a novel mechanisms or a parallel mechanism to papillomaviruses than similarity to other polyomaviruses (Spurgeon and Lambert, 2013). For example, in terms of virus receptors, MCPyV uses heparan sulphate and other glygosaminoglycans for attachment. Sialyated glycans typically involved in polyomaviral attachment serve as postattachment correceptors to MCPyV (Schowalter et al., 2011). Schowalter et al. (2012) observed a slow and asynchronous entry of MCPyV when examining entry into various cell lines.

Further knowledge about the life cycle is based on *in vitro* studies. In agreement with observations made on other polyomaviruses, T antigens play essential role in MCPyV replication (Spurgeon and Lambert, 2013). LT was shown to interact with Brd4 (Wang et al., 2012), a protein important for cellular growth control and cell cycle progression (Dey et al., 2000). LT binds Brd4 with its N-terminus within a replication complex localized at MCPyV ori. This binding is essential for replication, but it does not affect transcription (Wang et al., 2012). MCPyV genome can undergo integration into cell genome, which is an event linked to malignant transformation (see chapter 2.1.2.3.).

2.1.2.3. Pathogenicity

MCPyV productive infection has not been linked to any disorder so far, which is the case for some other polyomaviruses as well (see chapter 2.1.). MCPyV importance lies in the link to MCC, a neuroendocrine tumor of skin origin with high mortality and fast progress (Han et al., 2012). MCC is a rare cancer but its incidence is on the rise (Tilling and Moll, 2012). Merkel cells are localized in skin, they transduce mechanical stimuli to associated nerves (Lucarz and Brand, 2007). They are fully differentiated and post-mitotic, which doubts their role as cells of origin for MCC. Even though the MCC and Merkel cells share some endocrine markers (but not all of them), tumor progenitors are more probably some skin precursors. The actual progenitors are not known, but three adepts were hypothesized by Tilling and Moll (2012) – epidermal stem cells (precursors of Merkel cells), dermal stem cells and skin-derived precursors.

In MCC, MCPyV is integrated in concatemers and the genome sequence harbors mutations rendering it replication incompetent (Guastafierro et al., 2013). Integration is random in terms of the site in cell genome (Feng et al., 2008; Guastafierro et al., 2013; Martel-Jantin et al., 2012). MCPyV is integrated

clonally, which means the integration is in the same site not only in every tumor cells but also in metastases (Martel-Jantin et al., 2012), which affirms that integration must precede tumor development. The integrated genome often harbors mutations – mainly in LT sequence, where a truncating mutation commonly occurs after pRb binding domain. This mutation disrupts viral replication, but does not affect binding of tumor suppressors or the sequence coding for st antigen (Guastafierro et al., 2013). Another mutation – a deletion of 90 bp within the sequence of VP1 was observed in integrated genomes. Authors suggest this deletion is a consequence of integration, but do not suggest any impact on cells (Guastafierro et al., 2013) as structural proteins were not observed to play a role in malignantly transformed cells.

In MCC, no expression of structural proteins was observed (Pastrana et al., 2009), which supports the observation of productive infection disruption. The malignantly transformed cells usually express T antigens (Gustafsson et al., 2012), predicted oncoproteins. T antigens force cell into S phase and interact with oncosuppresors p53 and pRb. The truncating mutation described above results in disrupted viral replication but does not disrupt the affection of cell cycle regulation. The affecting of tumor suppressors by T antigens is probably the cause of cancer development (Spurgeon and Lambert, 2013). Surprisingly, in MCPyV cell transformation, st was shown to play a more important role than LT. Apart from its well described role in binding PP2A phosphatase, st acts in disruption of 4E-BP1 hyperphosphorylation by affecting mTOR signaling. Inhibition of 4E-BP1 hyperphosphorylation results in disruption of cap-dependent translation and was shown to be crucial for MCPyV malignant transformation (Kassem et al., 2008).

Even though a hit-and-run oncogenesis is also suspected for MCPyV (Spurgeon and Lambert, 2013), LT is indispensable for tumor maintenance at least in some MCC. Cell lines derived from MCC with LT expression were shown to be completely dependent on LT and underwent cell cycle arrest or died after LT knockdown (Houben et al., 2010). These results suggest that LT plays an essential role in MCC development and probably maintenance. MCPyV is found in 80 % of MCC (Feng et al., 2008; Martel-Jantin et al., 2012; Shuda et al., 2011) and Rodig et al. (2012) even suggest that when using a better detection method, every MCC will reveal harboring MCPyV genome. These data propose LT to be a perfect target for MCC treatment. There are attempts to develop such treatment based for example on siRNA (short interfering RNA) knockdown of LT (Hoque KM, 2012).

MCPyV appearance in skin and MCC doubtlessly prevails, but MCPyV was also described to be found in other locations, such as saliva, gut, urine, or blood (Dalianis and Hirsch, 2013). Its association with other tumors was also described such as chronic lymphocytic leukemia (Feltkamp et al., 2013) or non-small cell lung carcinoma (NSCLC). MCPyV DNA was found in lungs of NSCLC patients and the

virus was shown to affect gene expression involved in tumor development (Lasithiotaki et al., 2013), which implies that MCPyV role in other human malignancies should not be ignored.

2.1.2.4. Prevalence

MCPyV is a very common virus in human population, as demonstrated by the shedding of high titers of virions from skin of healthy individuals (Schowalter et al., 2010). A study detecting MCPyV on common environmental surfaces also agrees with the frequent MCPyV occurrence. Authors found MCPyV DNA in 75 % of tested samples, of which 5 % was resistant to DNase treatment and so potentially infectious (Foulongne et al., 2011). Unlike the skin swab samples testing (described above), when control skin biopsies from healthy individuals were examined, MCPyV DNA was found only in 10 – 20 % of samples (in contrast to MCC samples which contain MCPyV DNA in about 80 % of cases, see chapter 2.1.2.3.) (Martel-Jantin et al., 2012). Unlike BKV, which can be found in aborted fetuses and a vertical transmission is suspected to play an important role (see chapter 2.1.1.4.), MCPyV is not found in blood samples from neonates (Gustafsson et al., 2012) and vertical transmission is highly unlikely.

In terms of seroprevalence, results from various studies differ although they usually employ the same antigen (recombinant VP1 particles) for detection of antibodies in sera. Generally, the seroprevalence is rather high in population, ranging around 40 – 80 % (Kean et al., 2009; Pastrana et al., 2009) which is in accordance with the case for other human polyomaviruses (Kean et al., 2009). Although the MCPyV seroprevalence is equal or only slightly lower in healthy individuals compared to MCC patients, the titers of antibodies differ dramatically (Pastrana et al., 2009). Patients with MCC have much higher titers of neutralizing antibodies directed against MCPyV VP1, which suggest a strong reactivation of the virus (Pastrana et al., 2009) and an immunological failure preceding MCC development (Moore and Chang, 2010). This casts a shadow on the idea of vaccination to prevent MCPyV infection and MCC development. Even though VLPs based on MCPyV structural proteins can be produced easily and were shown to mount strong neutralization antibody response after vaccination of laboratory animals (Pastrana et al., 2009), patients suffering from MCC are the ones possessing the largest army of neutralizing antibodies.

2.1.3. Polyomaviral minor structural proteins

Minor structural proteins are late proteins, it means they are produced in late times post infection, after DNA replication started (Hyde-DeRuyscher and Carmichael, 1988). There are two minor proteins in every polyomavirus described so far (with a supposable exception for MCPyV, see chapter 2.1.2.1.) – VP2 and VP3. Both proteins share ORF, VP3 is a shorter version of VP2 (truncated at N-

terminus, Fig 4.1). One copy of minor protein is localized in every capsomer (a building unit of the capsid, composed of five VP1 proteins, Fig.2.3). Minor proteins are stabilized in the capsomer by hydrophobic interactions (Sapp and Day, 2009) and their function is much more than only structural.

Minor structural proteins are very important for polyomaviruses, as demonstrated by significantly diminished viability and infectivity when lacking or mutated (Huerfano et al., 2010; Mannová et al., 2002; Nakanishi et al., 2007; Nakanishi et al., 2006). The research of these proteins is challenging, they are not easy to work with due to several aspects. First, they are completely hidden inside the intact virion. They are inaccessible for antibodies until partial disassembly of the virion, which occurs in the ER (8 - 10 hours post infection) (Kuksin and Norkin, 2012). Second, they are significantly attracted to membranes, which can be demonstrated by prediction of transmembrane domains. There are three predicted transmembrane domains within the amino acid sequence of VP2 (2 domains in VP3, Fig. 4.11A). This fact complicates handling the proteins, since they often get stuck in membranes when expressed in an expression system. Third, minor proteins are considerably cytotoxic when expressed in cells alone (Huerfano et al., 2010), in result of which observation of their localization within the cell is significantly limited.

Considering the research limitations listed above, it is not surprising that very little is known about polyomaviral minor proteins. Majority of reported results are based on observations of two model polyomaviruses – SV40 and MPyV. These data represent the only way to create at least a rough picture of function and importance of minor proteins in polyomaviruses. There is a lot to be done in research of minor proteins.

Based on scarce results concerning their function, minor proteins obviously posses additional functions to the structural one. They were proven to be indispensable in several phases of the infection process. After viral entry, the first phase where minor proteins are necessary is trafficking through ER. Polyomaviruses are believed to be dependent on trafficking through ER on their way from cell surface to the nucleus (Sapp and Day, 2009). SV40 was shown to partially disassemble in the ER while colocalizing with protein disulphide isomerase (PDI) (Kuksin and Norkin, 2012), a member of the ERAD (endoplasmic-reticulum-associated protein degradation) pathway. SV40 infection was shown to be dependent not only on the ERAD pathway, but also on additional ER mechanism, the protein folding machinery (Schelhaas et al., 2007). This evidence strongly supports the involvement of ER in the trafficking of polyomaviruses. Even though the escape from the ER is still very unclear, minor proteins seem to play an important role in breaking through the ER membrane. MPyV and SV40 were both shown to be partially disassembled in the ER (Kuksin and Norkin, 2012) or after incubation at conditions similar to ER (Rainey-Barger et al., 2007). This

disassembly leads to exposition of minor proteins on the viral surface (Kuksin and Norkin, 2012; Rainey-Barger et al., 2007). These proteins have high affinity to membranes, with the ability (at least in case of VP2) to permeabilize them (Rainey-Barger et al., 2007). Minor proteins are resistant to alkaline extraction (Rainey-Barger et al., 2007), which supports the idea of their transmembrane localization (together with transmembrane domain predictions, Fig.4.11A) and possibly their classification as viroporins (Huerfano et al., 2010). They were observed on destroyed membranes of ER and mitochondria after cell lysis (Parton and Howes, 2010). In case of SV40, minor proteins were shown indispensable for cell lysis, with accumulation of viruses under cell membrane when their function was inhibited. Minor proteins of SV40 (unlike VP1 protein) were associated with membranes when expressed in *E.coli*, expression of VP3 resulted in bacterial lysis (Daniels et al., 2006). Both minor proteins of MPyV also associate with membranes, but unlike SV40, VP2 was shown to be able to perforate membranes (Rainey-Barger et al., 2007).

In SV40, a subsequent phase (transfer from cytoplasm to the nucleus) also relies on minor proteins (Nakanishi et al., 2007; Rainey-Barger et al., 2007). The importance lies particularly on the interaction between the major and minor structural proteins (Nakanishi et al., 1996; Nakanishi et al., 2006) and on the binding of heterodimer of importin α2/β (Nakanishi et al., 2002). Although all three structural proteins (VP1, VP2, VP3) contain NLS, only NLS of VP3 is sufficient and necessary for importin binding and nuclear entry (Nakanishi et al., 2002). On the other hand, minor proteins of MPyV do not contain NLS and when expressed individually, they are not transferred to the nucleus. During infection, their transfer into the nucleus is completely dependent on VP1 binding (Forstová et al., 1993).

Minor proteins are structural proteins, they form and stabilize viral capsid. Major structural protein VP1 forms pentamers, building units of the capsid. Ca^{2+} ions were shown to be important for capsid stabilization when assembled (Li et al., 2003), minor proteins are indispensable for its assembly. Pentamers *in vitro* are unable to form capsids under physiological conditions or they form tubular structures and smaller capsids at altered pH. If minor proteins are added, pentamers assemble into capsids of correct size under various conditions (Kawano et al., 2006). Concerning DNA packaging during virion assembly, data from SV40 and MPyV differ. Minor proteins of SV40 are longer than in MPyV, the additional C-terminal 40 amino acid sequence is homological to histones and binds DNA (Clever et al., 1993). Clever et al. (1993) suggest that multiple minor proteins could bind into viral minichromosome and help to package it in a way similar to histone H2A. Minor proteins of MPyV cannot play role in DNA packaging but they are hypothesized to bind to histones during assembly (Forstová et al., 1993).

Major structural protein of polyomaviruses (VP1) is able to self-assemble into artificial viral capsids or so called virus-like particles (VLPs) when expressed in an expression system (Salunke et al., 1986). When minor proteins VP2, VP3 or both are coexpressed with VP1 protein, they incorporate into the particles as well. One of the expression systems suitable for preparation of such particles is a baculoviral expression system, where successful assembly was observed (Forstová et al., 1993). Expression and purification of recombinant minor proteins are usually a complicated issue due to their affinity to membranes (as mentioned above) and cytotoxicity (Huerfano et al., 2010). Coexpression of minor proteins with VP1 and their incorporation into VLPs is thus an advantageous approach to ensure expression of minor proteins in high yields and their easy purification.

As mentioned above, there are additional roles for minor proteins in infection apart from the structural role. A change in their posttranslational modification (myristoylation on N-terminus of VP2 protein) results in markedly diminished infectivity (Mannová et al., 2002). VP2 is also necessary for posttranslational modifications of VP1 (Forstová et al., 1993). Taken together, minor proteins are indispensable for infection and their research is important, not only in terms of understanding polyomaviral life cycle. Minor proteins found their application also in using VLPs as a delivery system in gene therapy. Fusion of a therapeutic gene with minor protein and integration of the fusion product into VLPs proved efficient in treating cells (Inoue et al., 2008).

2.2. Monoclonal antibodies

Antibodies naturally occur in organisms as components of humoral immunity. They are secreted proteins consisting of two types of chains – shorter light chain (2 immunoglobulin domains, 25 kDa) and longer heavy chain (4 – 5 immunoglobulin domains, 50 – 75 kDa) (Fig.2.7). N-termini of both heavy and light chains contain a variable region – amino acid sequence unique for each B-lymphocyte clone, which binds antigen (Bartůňková and Hořejší, 2009).

In terms of laboratory use, two kinds of antibodies are recognized – monoclonal antibody and polyclonal antibody. Monoclonal antibody is a uniform entity, antibodies are produced by single B-lymphocyte clone. The affinity, isotype, binding site of each antibody molecule is identical. Polyclonal antibody is a collection of antibodies directed against one antigen but usually not the same epitope. Antibodies in this collection differ in isotypes, affinity and binding sites. Monoclonal antibodies are usually more complicated in terms of preparation (see chapter 2.2.2.), but their use is better reproducible (Howard and Bethell, 2009).

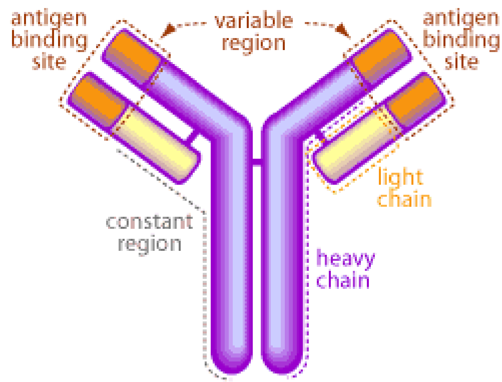


Fig.2.7: Scheme of antibody, heavy chains are shown in violet, light chains are shown in yellow. Variable regions (amino acid sequences unique for each B-lymphocyte clone) form the antigen binding site. Adopted from *http://www.scq.ubc.ca/mucosal-immunity-and-vaccines/*.

2.2.1. Application

Since the development of monoclonal antibodies preparation by hybridoma method in 1975 (see chapter 2.2.2.), monoclonal antibodies found their application in many fields. They are very stable, very specific and sensitive at the same time, which determines them for use in various sensitive tests. The application is not only in science - for example for detection or purification of various compounds. Monoclonal antibodies are also useful in medicine, where they serve in treatment of various diseases including cancer (Howard and Bethell, 2009).

2.2.2. Hybridoma method

Even though recombinant methods were developed for monoclonal antibodies preparations, hybridoma method is still being used nowadays thanks to its relative simplicity, low cost and long viability of immortalized hybridomas (Gorny, 2012).

The hybridoma method was developed in 1975 by Köhler and Milstein. In brief, the method proceeds as follows: 1. Laboratory animal (usually a mouse) is immunized repeatedly with the antigen of interest. 2. Mouse B-lymphocytes are fused with immortalized metabolically deficient myeloma cells using polyethylenglycol (or formerly used sendai virus). 3. Fusion products of interest (combining a myeloma and a B-lymphocyte) are selected on HAT medium profiting from myeloma metabolic deficiency and B-lymphocyte morality (usually supported by selection antibiotics because myelomas possess antibiotic resistance which is transmitted to fusants). 4. Hybridomas are tested for production of antibody of interest and cloned till a uniform population is achieved (Howard and Bethell, 2009).

3. Material and methods

3.1. Material

3.1.1. Machines

3130 Genetic Analyzer Sequenator

3K30 Centrifuge, 12171, 12154-H, 19776-H rotors (Sigma)

ABBE Refractometer (Carl Zeiss Jena)

Agarose electrophoresis horizontal apparatus multiSub Mini (Cleaver)

BX-60 Fluorescence Microscope (Olympus)

CO_2 thermostat (Forma Scientific)

Duomax 1030 Shaker (Heidolph)

Gene Pulser Apparatus Electroporator (Bio-Rad)

Genie 2 Vortex (Scientific Industries)

Helios β Spectrophotometer (Thermo Electron)

Inverted Light Microscope (Carl Zeiss Jena)

JEOL JEM-1011 Electron Microscope

Laminar Biohazard Cabinet (Forma Scientific)

LSR II Flow Cytometer (BD)

Mastercycler EPgradient S PCR Cycler (Eppendorf)

Microfuge Lite 16 Microcentrifuge, A46544 rotor (Beckman)

Microfuge R Microcentrifuge, F241.5 rotor (Beckman)

Mini-PROTEAN Tetra Cell apparatus for SDS-PAGE and Western blotting (Bio-Rad)

MiniSpin plus Microcentrifuge, IL 016 rotor (Eppendorf)

MSE Centrifuge with swing out rotor (MSE)

ND – 1000 Spectrophotometer (NanoDrop)

Olympus CK40 Inverted Light Microscope (Olympus)

Optima TM L-90K Ultracentrifuge - SW 28 and SW 41 rotors (Beckman)

Orbi-Safe TS Shaking Incubator (Gallenkamp)

Orbital Shaker Shaking Incubator (Forma Scientific)

PCR Cabinet (ESCO)

Shaker 30 (Labnet)

Soniprep 150 Sonicator (Schoeller)

SUB Water Bath (Grant)

TCH 100 Thermostat (laboratorní přístroje Praha)

Universal 320R Centrifuge (Schoeller)

UP50H Sonicator (dr. Hielscher)

UV Transluminator (BioLum)

VELP Scientific Vortex (P-LAB a.s.)

3.1.2. Chemicals

2-Mercaptoethanol (Arkema)

35% Hydrochloric Acid (Lach:ner)

70% Perchloric Acid (Lachema)

96% Ethanol (Lach:ner)

Acetic Acid (Penta)

Acetone (Lach:ner)

Acrylamide (Serva)

Agarose I^{TM} (Amresco)

Albumin from Bovine Serum (Sigma)

Alpha-D-Glucose (Serva)

Amonium Persulfate (Serva)

Ampicillin (BIOTIKA)

Aprotinin (Sigma)

Bayol F Paraffin Oil (Serva)

Bisacrylamide (Serva)

Boric Acid (Serva)

Bromphenol Blue (Výzkumný ústav přírodních léčiv, Praha 9)

Calcium Chloride (Sigma)

Cellfectine II Reagent (Invitrogen)

Caesium Chloride (Serva)

Chloroform (Lachema)

Coomassie Brilliant Blue G250 (CBBG) (Serva)

Deoxyribonucleoside Triphosphates – dATP, dTTP, dCTP, dGTP (Fermentas)

Dimethylsulfoxid – DMSO (Sigma)

Ethylendiamintetraacetate Disodium – EDTA (Serva)

Freund´s Adjuvant (Sigma)

Gelatin from porcine skin (Sigma)

Glycerol (Penta)

Glycine (Serva)

HIS-Select® Nickel Affinity Gel (Sigma)

Hydrogen Peroxide (Sigma)

IPTG dioxane free (Fermentas)

Isopropylalcohol (Lach:ner)

Luminol (Sigma)

Magnesium Chloride (Lachema)

Magnesium Sulfate (Lach:ner)

Methanol (Lach:ner)

N,N,N´,N´ - Tetramethylethylendiamin – TEMED (Sigma)

Nutrient Agar N°2 (Biolife)

Paraformaldehyde – PFA (Sigma)

p-Coumaric Acid (Sigma)

Peptone Bacteriological (Biolife)

Phenol (Sigma)

Potassium Chloride (Lachema)

Protease Inhibitor Cocktail Tablets EDTA-free (Roche)

SeaPlaque Agarose (FMC Bioproducts)

Sodium Chloride (Lach:ner)

Sodium Dodecyl Sulphate – SDS (Sigma)

Sodium Hydroxide (Penta)

Sucrose (Lach:ner)

Tris(hydroxymethyl)aminomethane TRIS (Serva)

Triton X-100 (Serva)

X-gal (Fermentas)

Yeast Extract (IMUNA)

3.1.3. Cell lines

Sf9 Insect cell line derived from ovaries of *Spodoptera frugiperda*. The cell line originates from American Type Cell Collection (ATCC CRL 1711).

SP 2/0 Mouse clone cell line derived from B-cell myeloma of BALB/c mouse. The cell line was kindly provided by Research Group of Developmental Biology (Department of Cell Biology, Charles University). SP 2/0 myelomas posses resistance against gentamicin and are producers of interleukin 6.

3.1.4. Viruses

WT Bac Wild type baculovirus (kindly provided by Max D. Summers, Texas Agricultural Experiment Station)

R Bac VP1/2 BKV Recombinant baculovirus producing VP1 protein of BK virus (under polyhedrin promoter) and VP2 protein of BK virus (under p10 promoter). Expressed proteins spontaneously form VLPs (Hrušková and Stančíková, unpublished data).

R Bac VP2 BKV Recombinant baculovirus producing VP2 protein of BK virus fused with His-tag (under polyhedrin promoter) (K. Podolská, diploma thesis, 2008).

R Bac VP1/2 MCPyV Recombinant baculovirus producing VP1 protein of MCPyV (under polyhedrin promoter) and VP2 protein of MCPyV (under p10 promoter). Expressed proteins spontaneously form VLPs. Baculovirus was prepared as part of this diploma thesis.

3.1.5. Bacteria

***E. coli* XL1 blue** [recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac F´proAB lacIqZΔM15 Tn10 (Tetr)] (Stratagene)

***E. coli* BL21(DE3)** F^{-} ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) (Department of Genetics and Microbiology Collection)

***E. coli* DH10Bac™** F– mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara leu) 7697 galU galK λ– rpsL nupG/ pMON14272/pMON7124 (Invitrogen)

3.1.6. Vectors

pET29b a plasmid vector carrying N-terminal S-tag and C-terminal His-tag (optional), plasmid also contains a kanamycin resistance gene, T7 promoter and terminator (Novagen)

c734A a plasmid carrying the whole sequence of BK viral genome (cloned in PstI and BamHI restriction sites), used as PCR template for preparation of truncated VP2 protein of BK virus (kindly provided by Dr. Kristina Dorries, University of Würzburg)

pFastBac Dual a plasmid vector suitable for cloning genes of interest for protein production in bac-to-bac system, plasmid contains two promoters (PH and p10) under which genes of interest can be cloned, plasmid contains ampicilin and gentamicin resistance genes and transposition site (Tn7L and Tn7R) for site-specific transposition (Invitrogen)

pwm a plasmid carrying sequence of VP1 of MCPyV, used as PCR template for preparation of VP1 gene for bac-to-bac system (Addgene plasmid 22515, Pastrana et al (PLoS Pathog. 2009 Sep . 5(9):e1000578))

ph2m a plasmid carrying sequence of VP2 of MCPyV, used as PCR template for preparation of VP2 gene for bac-to-bac system (Addgene plasmid 22518, Pastrana et al (PLoS Pathog. 2009 Sep . 5(9):e1000578))

pMON7124 a helper plasmid in DH10Bac bacteria, contains a tetracycline resistance gene and helps with transposition from pFastBac plasmid to pFastBac bacmid (Invitrogen)

bMON14272 a shuttle vector (bacmid) in DH10Bac bacteria, contains a kanamycin resistance gene and LacZα gene (disruption of this gene occurs after transposition as the sequence contains Tn7 transposition site)

3.1.7. PCR Primers

Bac-to-bac system – cloning of VP1 of MCPyV (template: pwm plasmid)

VP1 forward: 5'-ACT TGA **CTC GAG** *ATG GCC CCG AAG CGC AAG*-3'
VP1 reverse: 5'-GTC CAG **GCT AGC** *TCA TAG CTC CTG CGT CTG*-3'

Forward primer includes a **restriction site** for XhoI restrictase (C↓T C G A G). Reverse primer includes **restriction site** for NheI restrictase (G↓C T A G C). The sequence complementary to VP1 sequence is shown in *italics*.

Bac-to-bac system – cloning of VP2 of MCPyV (template: ph2m plasmid)

VP2 forward: 5'-ACT TGA **GTC GAC** *ATG GGC GGG ATC ATT ACC*-3'
VP2 reverse: 5'-GGC CAG **CTG CAG** *TCA GAG ATG AAT GAT TGA*-3'

Forward primer includes a **restriction site** for SalI restrictase (G↓T C G A C). Reverse primer includes **restriction site** for PstI restrictase (C T G C A↓G). The sequence complementary to VP2 sequence is shown in *italics*.

Bac-to-bac system – sequencing of VP1 of MCPyV

VP1 forward: 5'-TCG TCG GAT TCC TGT TCA AG-3'
VP1 reverse: 5'-AGT GGA CGT TGA TCA GGC TG-3'

Primers were used as additional pair for sequencing (to achieve complete sequencing data). The sequences are complementary to VP1 sequence.

Cloning of truncated VP2 (tVP2) of BK virus (template: c734A plasmid)

tVP2 forward: 5'-CTA CGA **AGA TCT** T*AT GGG TGC TGC TCT AGC*-3'
tVP2 reverse: 5'-CGG TTG **GTC GAC** *AGT TCT TTG ATT AGC ACC*-3'

Forward primer includes a **restriction site** for BglII restrictase (A↓G A T C T). Reverse primer includes **restriction site** for SalI restrictase (G↓T C G A C). The sequence complementary to tVP2 sequence is shown in *italics*.

3.1.8. Antibodies

3.1.8.1. Primary antibodies

Anti VP2/3 SV40 rabbit polyclonal antibody against VP2/3 SV40, dilution 1:500 (kindly provided by prof. Ariela Openhein)

Anti VP1 MCPyV (B41E5) mouse antibody (collection of culture media from hybridoma clone) against VP1 MCPyV (structural epitope), dilution 1:1 (P. Sauerová, diploma thesis, 2013)

Anti VP1 MCPyV (serum) mouse polyclonal serum against VP1 of MCPyV, dilution 1:750 (P. Sauerová, diploma thesis, 2013)

Anti His-tag rabbit polyclonal antibody against His-tag, used in dilution 1:100 (Santa Cruz Technology)

Anti S tag mouse monoclonal antibody against S-tag, dilution 1:1000 (Merck)

Anti GFP rabbit monoclonal antibody against GFP, dilution 1:500 (Abcam)

3.1.8.2. Secondary antibodies

Goat anti mouse – HRP goat polyclonal antibody against mouse immunoglobulins conjugated with horse radish peroxidase (HRP), used in dilution 1:1000 (Bio-Rad)

Goat anti rabbit – HRP goat polyclonal antibody against rabbit immunoglobulins conjugated with HRP, used in dilution 1:1000 (Bio-Rad)

Goat anti mouse – Alexa 488 goat polyclonal antibody against mouse immunoglobulins conjugated with Alexa fluor (488), used in dilution 1:1000 (Invitrogen)

Donkey anti rabbit – Alexa 488 donkey polyclonal antibody against mouse immunoglobulins conjugated with Alexa fluor (488), used in dilution 1:1000 (Invitrogen)

3.1.9. Fluorescent stains

Ethidium bromide (Calbiochem) 0.5 ng/ml

GelRed Nucleic Acid Stain (Biotium) used in dilution 1:10000

DAPI (4,6-diaminido-2-phenylindol) used in dilution 1:500 (in glycerol)

3.1.10. Enzymes

Restriction endonucleases (Fermentas)

- BglII
- SalI
- XhoI
- NheI
- PstI
- EcoRI

T4 DNA ligase (Fermentas)

Taq DNA polymerase (Fermentas)

High Fidelity DNA polymerase (Fermentas)

FastAP™ alkaline phosphatase (Fermentas)

3.1.11. Culture media

3.1.11.1. Bacterial media

SOC medium 2% bacteriological pepton, 0.5% yeast extract, 20mM glucose, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$

TPN medium 2% bacteriological pepton, 0.5% yeast extract, 0.5% NaCl

LB medium 1% bacteriological pepton, 0.5% yeast extract, 1% NaCl

2xYT medium 1.5% bacteriological pepton, 1% yeast extract, 0.5% NaCl

3.1.11.2. Cell culture media

1640 RPMI (for myelomas) 1640 RPMI medium (Sigma), 10% fetal bovine serum (FBS, Gibco), 1mM pyruvate (Gibco), 2mM glutamine (Sigma), 50µM β-mercaptoethanol (Arkema), antibiotics mix (see 3.1.12., Sigma)

HAT medium 1640 RPMI medium (Sigma), 10% FBS(Gibco), 1mM pyruvate (Gibco), 2mM glutamine (Sigma), 50µM β-mercaptoethanol (Arkema), antibiotics mix (see chapter 3.1.12., Sigma), HAT supplement (Gibco)

Freezing medium FBS (Gibco), 10% DMSO (Sigma)

Agarose medium 1.5% SeaPlaque Agarose (FMC Bioproducts), 0.5x TNM-FH insect medium (Sigma), 10% FBS (Gibco), antibiotics mix (see chapter 3.1.12., Sigma)

Insect medium with FBS TNM-FH insect medium (Sigma), 10% FBS (Gibco), 2mM glutamine (Sigma), antibiotics mix (see chapter 3.1.12., Sigma)

Insect medium (no FBS) TNM-FH insect medium (Sigma), antibiotics mix (see chapter 3.1.12., Sigma)

3.1.12. Antibiotics

Antibiotics mix for cell cultures 100x concentrated mix with penicillin (10000 units), streptomycine (10 mg) and amphotericine B (25 mg) (Sigma)

Kanamycin final concentration 50 µg/ml (Sigma)

Ampicillin final concentration 100 µg/ml (Biotika)

Tetracycline	final concentration 10 µg/ml (Sigma)
Gentamicin	final concentration 7 µg/ml (Sigma)
Geneticin G418	final concentration 200 µg/ml or 500 µg/ml (Sigma)

3.1.13. Markers

DNA electrophoresis

O' GeneRuler™ 1 kb Plus DNA Ladder (ready-to-use) (Sigma) was applied in DNA electrophoresis (3 µl). List of fragments present in the marker is shown on figure 3.1.

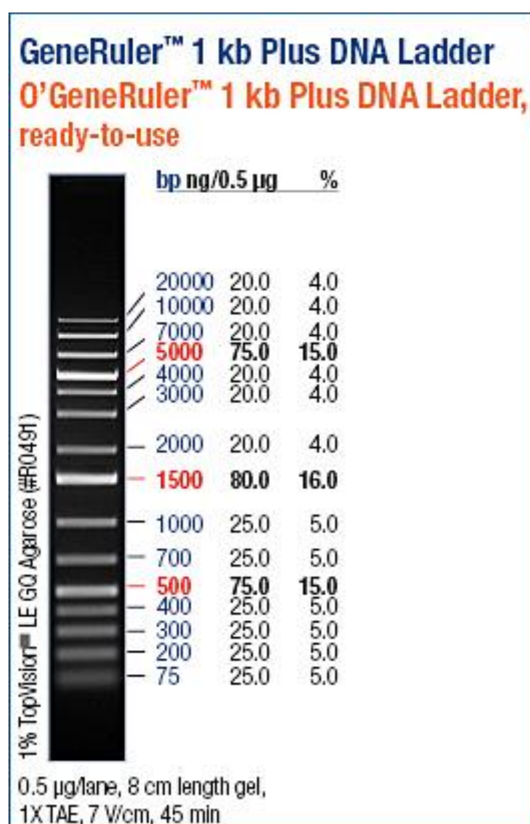


Fig.3.1: Fragments of O'GeneRuler™ 1 kb Plus DNA Ladder (Sigma). Adapted from http://www.thermoscientificbio.com/nucleic-acid-electrophoresis/ogeneruler-1-kbplus-dna-ladder-ready-to-use-75-20000-bp/

Protein electrophoresis

SDS Molecular Weight Standard mixture (SDS7B, Sigma) was applied in SDS PAGE (10 µl). The mixture contains 7 prestained proteins, molecular weights are listed in table 3.1.

Prestained Protein	Native* Mol. Wt. (subunit)
α_2-Macroglobulin from human blood plasma	180,000
β-Galactosidase from *E. coli*	116,000
Lactoferrin from human milk	90,000
Pyruvate Kinase from chicken muscle	58,000
Fumarase from porcine heart	48,500
Lactic Dehydrogenase from rabbit muscle	36,500
Triosephosphate Isomerase from rabbit muscle	26,600

Table 3.1: Proteins and their molecular weights present in SDS Molecular Weight Standard mixture (Sigma). Adapted and modified from http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/sds7b2bul.Par.0001.File.tmp/sds7b2bul.pdf

Spectra^{TM} Multicolor Broad Range Protein Ladder (Fermentas) was applied in Western blots (10 µl). List of fragments present in the marker are shown in Fig. 3.2

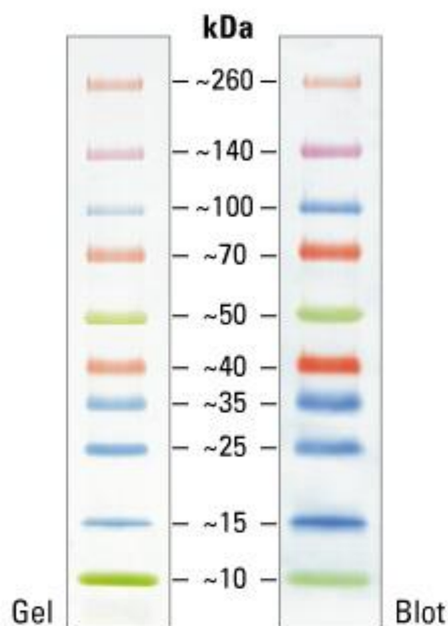


Fig. 3.2: Protein fragments molecular weight (in kDa) present in molecular marker used for Western blotting. Adapted from *http://www.piercenet.com/browse.cfm?fldID=71829CE6-F15D-F722-FC02-E2F53427A8A4*

3.1.14. Solution contents

B buffer	10mM Tris-HCl (pH 7.4), 150mM NaCl, 10µM $CaCl_2$
Coomassie Blue Solution	0.5% CBBG, 3.5% $HClO_4$
Fixing solution (SDS PAGE)	5% $HClO_4$, 50% methanol

Blocking solution (IF) 0.25% gelatine, 0.25% BSA in PBS

Blocking solution (FACS) 0.25% gelatine, 5% FBS in PBS

Blotting buffer 25mM Tris-HCl, 185mM glycine, 20% methanol

Bradford reagent 0.002% CBBG, 5% ethanol, 10% H_3PO_4

Electrophoresis buffer 250mM Tris-HCl, 1.92M glycine, 1% SDS

Fixing solution (IF) methanol:acetone = 1:1

Laemmli buffer (5x) 50mM Tris-HCl (pH 6.8), 5% SDS, 50% glycerol, 25% β-mercaptoethanol, 0.005% bromphenol blue

Luminol A: Tris-HCl (pH 8.5), 0.005% luminol, 0.006% coumaric acid
B: Tris-HCl (pH 8.5), 0.001% hydrogen peroxide

PBS 0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4

RIPA buffer 150mM NaCl, 5mM EDTA, 50mM Tris-HCl (pH 7.4), 0.05% NP-40, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS

Solution I (plasmid pur.) 50mM glucose, 25mM Tris-HCl (pH 8), 10mM EDTA, 0.0001% RNase A

Solution II (plasmid pur.) 0.2M NaOH, 1% SDS

Solution III (plasmid pur.) 29.5% CH_3COOH, 3M KAc (pH 5.2)

TBE (0.5x) 0.54% Tris-HCl, 0.275% boric acid, 0.01M EDTA (pH 8)

3.2. Methods

3.2.1. Work with tissue cultures

3.2.1.1. Passaging of insect cells (Sf9 cell line)

From confluent Petri dishes, cells were scrubbed away and transferred to a sterile tube. Cell suspension was diluted in fresh medium with FBS and well resuspended. The suspension was distributed to sterile Petri dishes – 5 ml in a Ø 6 cm dish or 10 ml in a Ø 10 cm dish. Culture was cultivated in a cell incubator at 27°C.

3.2.1.2. Cell counting in Bürker cell counter

Approximately 20 µl of cell suspension was placed in a cell counter. Cells were counted in 32 squares (16 and 16 diagonally). Number of cells in 1 ml was determined using the formula:

$$\frac{cells}{ml} = \text{Ø}\, number\ of\ cells\ in\ a\ square * 2.5 * 10^5$$

3.2.1.3. Infection of insect cells (Sf9 cell line)

Cells grew to 50 – 70 % confluence in medium with FBS. Medium was removed and cells were washed carefully with fresh medium (no FBS). Baculoviral inoculum was added – 1 ml (Ø 10 cm dish) or 0.5 ml (Ø 6 cm dish) and dishes were incubated on a shaker for 60 minutes. Afterwards, 9 ml (Ø 10 cm dish) or 4.5 ml (Ø 6 cm dish) of medium with FBS was added to dishes. Culture was cultivated in a cell incubator at 27°C for 72 hours.

3.2.1.4. Transfection of insect cells

Cells Sf9 were passaged in a 6-well dish so as to obtain 10^6 cells per well, no antibiotics were present in medium. When cells were attached, medium was replaced with fresh one with 1.5% FBS. Medium (no additives, 100 µl) was mixed with Cellfectin II (8µl) and bacmid sample (1 µl), mixture was incubated for 20 minutes and then dripped carefully on cells. Three hours after transfection, medium was replaced with medium with FBS and cells were incubated at 27°C in an incubator.

3.2.1.5. Fixing cells for immunofluorescence assay

Cells (Sf9 cell line) were cultivated and infected on round slides in dishes. Culture media was carefully removed and cells were washed with PBS. Cells were treated for 5 minutes with a solution of acetone and methanol (1:1) to fix. Afterwards, cells were washed with PBS twice.

3.2.1.6. Indirect immunofluorescent staining

Fixed cells were blocked in blocking solution (0.25% gelatine and 0.25%BSA in PBS) for 30 minutes. Primary antibody was applied for one hour. Cells were washed in PBS three times (10 minutes each washing). Secondary antibody was applied for 30 minutes. Cells were washed in PBS three times (10 minutes each washing). Finally, slides were washed in distilled water, dried and applied into a 4 µl drop of 50% glycerol with DAPI (for DNA visualisation).

3.2.2. Antibody preparation

3.2.2.1. Mice immunisation

Mice need to be immunised as the first step of antibody preparation. Immunogen (protein of interest with adjuvants) was injected intraperitoneally or expressed in the mouse (gene gun method).

3.2.2.1.1. Protein solution (BKV unique part)

Mice immunisation was performed by RNDr. Alena Morávková, Ph.D.

Mice were immunised with 100 µl of protein solution prepared by M. Verdánová (diploma thesis, 2011; concentration 0.23 mg/ml) and 100 µl of Complete Freund´s Adjuvant (CFA). Dose was stirred properly and injected peritoneally. Immunisation was repeated three times (after two weeks and four weeks). Six weeks after the first immunisation, blood sample was taken from *vena caudalis*. Blood serum was tested by dot blot for antibody presence. Mouse chosen for hybridoma fusion was boosted with the same dose intravenously three days before the fusion.

3.2.2.1.2. Gene gun and cell lysates (MCPyV VP2)

Mice immunisation was performed by RNDr. Michal Šmahel, Ph.D. (gene gun) and RNDr. Alena Morávková, Ph.D. (cell lysates). Antigens were prepared by Mgr. Martina Kojzarová (M. Kojzarová, unpublished data).

Four mice were immunised by gene gun method with modified ph2m plasmid (3 doses, concentration 1000 ng/µl). Modification of plasmid (deletion of GFP coding region) was performed by Mgr. Martina Kojzarová. Immunisation efficiency was checked by testing mouse blood serum on dot blot. According to results, efficiency of immunisation was rather weak. Two mice (those with stronger antibody production) were further immunised with cell lysates.

BALB 3T3 cells (syngenic to immunised mice) were transfected with the modified ph2m plasmid (described above). Cells were lysed natively by repeated freezing in liquid nitrogen and thawing. Preparation of cell lysates was performed by Mgr. Martina Kojzarová. Mice were immunised intraperitoneally with cell lysates (3 doses, 100 µl of lysates + 100 µl of CFA).

3.2.2.2. Hybridoma fusion

3.2.2.2.1. Myeloma cells

Myeloma cells Sp2/0 were stored in liquid nitrogen. Cells were thawed, diluted 1:5 in RPMI 1640 medium for myelomas (see chapter 3.1.11.2. for contents) and distributed to cultivation flasks. Cells grew for several days and then were used for fusion.

3.2.2.2.2. Feeder cells

Feeder cell layer is necessary for ensuring convenient chemical environment for hybridoma growth. A mix of peritoneal mouse cells, mainly peritoneal macrophages, was used as feeder cells. Feeder cells were obtained by peritoneal rinsing. Mouse was sacrificed by cervical dislocation and the body was washed in 96% ethanol. Further work was performed in a biohazard cabinet. Abdominal skin was removed, peritoneal cavity was rinsed repeatedly by 5 ml of HAT medium (see chapter 3.1.11.2. for contents). Obtained medium with peritoneal cells was diluted to 50 ml and distributed into five 96-well plates. This procedure was performed one day prior to cell fusion or cloning.

3.2.2.2.3. Spleen extraction

Mouse was sacrificed by cervical dislocation, body was washed in 96% ethanol. Further work was performed in a biohazard cabinet. Skin was removed and spleen was extracted from abdominal cavity with sterile surgical instruments. Spleen was cut with sterile scissors in RPMI 1640 medium (no additives) and filtered through nylon.

3.2.2.2.4. Fusion

Myeloma cells were washed (centrifuged at 90 g, 10 min and resuspended in fresh medium) three times and so were spleen cells. Concentration of cells was determined in Bürker cell counter. Cell mixture in 5:1 ratio (spleen cells : myelomas) was prepared and washed once more. Supernatant was removed and 1 ml of PEG 3350 was gradually added into the mixture over one minute´s time. During the following minute, 1 ml of HAT medium was slowly added into the mixture. Afterwards, 8 ml of HAT medium was added. Newly formed hybridomas were centrifuged at 90 g, 10 min, resuspended in fresh HAT medium and distributed into five 96-well plates on a feeder cells layer.

3.2.2.3. Hybridoma maintenance

Hybridomas were cultivated in 96-well plates, in 200 µl of HAT medium per well.

3.2.2.3.1. Geneticin treatment

Three days after fusion, culture medium was replaced with HAT medium with 200 ng/µl of geneticin. Geneticin helps to remove feeder cells and other undesirable fusion products. Geneticin also protects hybridomas against prokaryotic contamination. After three days, medium with geneticin was removed and fresh HAT medium was added to cells.

3.2.2.3.2. Cloning

Culture media from wells were tested and the most specific antibody producing colonies were cloned. Colonies from one well were resuspended properly and counted using a Bürker cell. Cells were diluted in an adequate amount of HAT medium to achieve concentration of one cell per well after cloning (limited dilution). Cell suspension was distributed into 96 wells (one 96-well plate) on a feeder cells layer.

3.2.2.3.3. Storing

Positive hybridoma colonies, which were not cloned, were stored in liquid nitrogen. Colonies from one well were resuspended properly in 400 µl of HAT medium and transferred into a microtube. Cells were centrifuged (500 g, 10 min). HAT medium was removed, cells were resuspended in 200 µl of freeze medium (FBS, 10% DMSO). Cell suspension was transferred into a cryotube and stored in deep freeze (-80°C) for 24 hours in a freeze box. Then, cryotubes were transferred to liquid nitrogen container.

3.2.2.4. Hybridoma testing

Hybridomas produce antibody into the culture medium. Culture media from populated wells were collected and replaced with fresh HAT medium. The collected media were tested for the presence of specific antibody. To reduce bias, test results were evaluated on a comparative basis (related to negative and positive controls).

3.2.2.4.1. FACS

Testing was largely carried out by FACS analysis. Sf9 cells infected with a recombinant baculovirus producing the protein of interest (VP2 from MCPyV, VP2 fused with His-tag from BKV or VP1 and VP2 from BKV) were used as antigens. Infected cells were collected 72 h.p.i. when sufficient amount of the protein of interest was produced. Cells were washed in PBS, centrifuged at 90 g for 10 minutes. Cells were fixed in cold 70% ethanol for 5 minutes. Cells were centrifuged at 500 g, 10 minutes,

ethanol was removed and cells were washed in PBS and centrifuged again. Cells were resuspended in blocking solution (see chapter 3.1.14. for contents) and blocked for at least 30 minutes. Cell concentration was determined in Bürker cell counter.

Blocked cells were distributed into 96-well plates with pre-introduced primary antibodies (hybridoma culture media collected earlier). The amount of cells was approximately $2 \cdot 10^5$ per well. After one hour, plates were centrifuged at 90 g, 10 min in a refrigerated centrifuge. Supernatant was removed by careful splashing off. Cells were washed three times in PBS (identical centrifugation between washings). Secondary antibody (fused with Alexa Fluor 488) diluted in blocking solution (1:1000) was added to wells and cells were incubated for 30 minutes. Centrifugation and two washings in PBS followed.

Cells were resuspended in 50 µl of PBS and measured on FACS. Measuring was performed by RNDr. Alena Morávková, Ph.D.

3.2.2.4.2. Dot blot

Only overgrown colonies or colonies cultivated in larger scale were tested by dot blot. Sf9 cells infected with a recombinant baculovirus producing the protein of interest (VP2 from MCPyV or VP2 from BK virus) were used as antigens. Infected cells were collected 72 h.p.i. when sufficient amount of the protein of interest was produced. Cells were washed in PBS, centrifuged at 90 g for 10 minutes. Cells were lysed in RIPA buffer (see chapter 3.2.5.3. for details of the procedure) and 2 µl drops of suspension were applied on nitrocellulose membrane. Membrane was blocked in blocking solution for 30 minutes and primary antibodies (hybridoma culture media) were applied on membrane and incubated for one hour. The rest of the procedure was identical to standard dot blot (chapter 3.2.5.4.).

3.2.3. Work with DNA

3.2.3.1. Plasmid isolation by Qiagen kit

Isolation was performed according to manufacturer´s instructions. Bacterial culture was cultivated overnight and was centrifuged in a refrigerated centrifuge in 5 ml aliquots (4000g, 10 min). Each bacterial aliquot was resuspended in 250 µl of P1 buffer and transferred to a microtube. Buffer P2 (250 µl) was added and microtube was inverted several times. Buffer N3 was added (350 µl) and sample was mixed well by repeated microtube inverting. Sample was centrifuged in a refrigerated centrifuge (20000 g, 10 min) and supernatant was applied on a column (QIAprep spin column).

Column with sample was centrifuged to remove buffers (DNA was bound on the membrane) and washed with PB buffer (500 µl) and PE buffer (750 µl). DNA was eluted by 50 µl of EB buffer.

3.2.3.2. Plasmid isolation by alkaline method

Bacterial culture (500 ml) was cultivated overnight and was centrifuged in a refrigerated centrifuge in 50 ml aliquots. Supernatant was removed and pellets were resuspended in 20 ml of solution I. Solution II (40 ml) was added to the sample, mixed by inverting the tube and incubated for 10 minutes. Solution III (30 ml) was added to the sample, mixed by inverting the tube and incubated for 10 minutes on ice. The sample was centrifuged in a refrigerated centrifuge (20000g, 15 min) and supernatant was transferred to a fresh tube. Isopropanol (0.6 volume) was added to the sample, stirred well on vortex and incubated for 10 minutes. DNA was purified by phenol-chloroform method and precipitated with 96% ethanol.

3.2.3.2.1. Plasmid minipreparation

Plasmid preparation by a modified alkaline method (minipreparation) was applied in case of testing several bacterial colonies. Bacterial monocolony (cultivated on agar plates) was inoculated in 700 µl of culture medium with selective antibiotics (in a microtube). Microtubes were cultivated overnight at 37°C in a shaker. In the morning, the microtubes were centrifuged (4000g, 10 min). Supernatant was removed and pellets were resuspended in 250 µl of solution I. Solution II (250 µl) was added to the samples, mixed by inverting the tube and incubated for 10 minutes. Solution III (200 µl) was added to the samples, mixed by inverting the tube and incubated for 10 minutes on ice. Sample was centrifuged in a refrigerated centrifuge (20000g, 15 min) and supernatant was transferred to a fresh tube. Isopropanol (500 µl) was added to the sample, stirred well on vortex and incubated for 10 minutes. DNA was precipitated with 96% ethanol.

3.2.3.2.2. Bacmid isolation

A modified alkaline method was used for bacmid (recombinant baculoviral genome, high-molecular DNA) isolation. Bacterial monocolonies (cultivated on agar plates) were inoculated in 5 ml of culture medium with selective antibiotics (kanamycin, gentamicin and tetracyclin) and were cultivated overnight at 37°C in a shaker. In the morning, 1.5 ml aliquots were centrifuged (4000g, 10 min). Supernatant was removed and pellets were resuspended in 300 µl of solution I. Solution II (300 µl) was added to the samples, mixed by inverting the tube and incubated for 5 minutes. Solution III (300 µl) was added to the samples, mixed by inverting the tube and incubated for 10 minutes on ice. Sample was centrifuged in a refrigerated centrifuge (20000 g, 15 min) and supernatant was transferred to a fresh tube with isopropanol (800 µl). Afterwards, the samples were centrifuged

(20000 g, 30 min), pellet was carefully washed with 70% ethanol and centrifuged again. Dried pellets were resuspended in ddH_2O.

3.2.3.3. Phenol-chloroform purification

To remove protein contamination from DNA sample (after plasmid isolation, restriction cleavage, PCR), phenol-chloroform purification was applied. The sample volume was measured and the same volume of phenol was added and stirred well on vortex. Sample was centrifuged (5 min, max. speed) and two layers parted. Upper layer was transferred to a new microtube and mixed with half volume of phenol and half volume of chloroform. Sample was again stirred and centrifuged and upper layer was transferred to a new microtube. The sample was mixed with the same volume of chloroform, stirred and centrifuged. Upper layer (containing purified DNA) was transferred to a new centrifuge and precipitated with 96% ethanol.

3.2.3.4. Ethanol precipitation

Ethanol precipitation was applied usually as the final step of DNA purification, in order to concentrate the sample. DNA sample was mixed with 0.1 volume of 3M sodium acetate and 3 volumes of cold 96% ethanol. Mixture was stirred properly using vortex and incubated at -20°C for one hour. Sample was centrifuged in a refrigerated centrifuge (20000 g, 25 min), pellet was washed in 70% ethanol and centrifuged (20000 g, 25 min). Pellet was dried and dissolved in ddH_2O.

3.2.3.5. Agarose electrophoresis

DNA samples were applied on 1% agarose gel to verify molecular weight of fragments. Agarose was dissolved in appropriate amount of 0.5 TBE buffer. Complete dissolution was reached when solution was heated. After cooling the solution was mixed with fluorescent dye for visualisation of DNA (ethidium bromide or GelRed Nucleic Acid Stain) and transferred to electrophoresis chamber equipped with electrophoresis comb. When the gel was ready, marker of molecular weight (3 µl) was applied in the first hole. DNA sample was mixed with 6 x Orange DNA Loading Dye (dilution 1:6, Fermentas) and applied in holes. Voltage (70 V) was applied and gel was photographed in UV transluminator at the end of electrophoresis.

3.2.3.6. Restriction cleavage with endonucleases

Restriction mix was prepared according to manufacturer's instructions (for each restriction endonuclease, Fermentas). It contained purified DNA (200 ng - 1 µg), restriction buffer, restriction endonuclease (1 – 10 units) and ddH_2O – total volume of 20 - 100 µl. Mixture was incubated for several hours or overnight in appropriate temperature (37°C in all cases).

3.2.3.7. Dephosphorylation

To avoid undesired religation of cleavage products, DNA ends were dephosphorylated after restriction cleavage. Dephosphorylation mix was prepared according to manufacturer's instructions for FastAP^{TM} alkaline phosphatase. It contained cleaved DNA (200 ng – 1 µg), FastAP^{TM} buffer, FastAP^{TM} alkaline phosphatase (1 unit) and ddH_2O – total volume of 50 - 70 µl.

Sample was incubated in 37°C for one hour, afterwards reaction was terminated by heating sample (75°C) for five minutes. Phenol-chloroform purification and ethanol precipitation of sample followed.

3.2.3.8. Ligation

Ligation mix was prepared according to manufacturer's instructions for T4 DNA ligase. Molar ratio of insert to vector was set to 3:1. Ligation mix contained purified DNA (200 ng of vector and ~40 ng of insert), T4 DNA ligase buffer, T4 DNA ligase (5-10 units) and ddH_2O – total volume of 20 µl. Sample was incubated at 22°C overnight.

3.2.3.8.1. Ladder ligation

To verify successful restriction cleavage of PCR products, ligation producing multiple-length fragments of PCR products (ladder ligation) was performed. Ligation mix contained purified DNA (100 ng), T4 DNA ligase buffer, T4 DNA ligase (2.5 - 5 units) and ddH_2O – total volume of 20 µl. Sample was incubated for 3 hours at 22°C.

3.2.3.9. PCR amplification

PCR amplification was performed in volume of 50 µl. PCR mix without template was prepared on ice, the mix was distributed to PCR microtubes and template DNA was added to each sample except negative control. PCR mix contained dNTPs (deoxy nucleoside triphosphates), primers (forward and reverse), template DNA, Thermo buffer, Taq polymerase (1 unit per reaction).

In case of bacmid verification (chapter 4.4.2.), High Fidelity Polymerase and respective buffer were used instead. The volume of PCR mix was set to 20 µl.

Appropriate temperature for primer annealing step was found for each primer pair. Volume of PCR mix was set to 20 µl. Temperature interval of 10°C was tested (in 10 reactions). Annealing temperature at which PCR product was specifically produced in high yield was chosen. In some cases,

yields were equal at all temperatures and so the combined products of primer testing were used for further cloning.

PCR program was set up as follows:

1) initial template denaturation	94°C	3 min
2) template denaturation	94°C	50s
3) primer annealing	set individually	50s
4) DNA synthesis	72°C	50s – 1min 20s (1000 bp ~ 1 min)
5) final DNA synthesis	72°C	7 min
6) cooling	4°C	not defined

Steps 2 - 4 were repeated 30 times.

3.2.3.9.1. Colony PCR

Colony PCR was performed in 20 µl reaction mixtures in order to verify presence of desired DNA sequence in a crude bacterial lysate. Bacteria were cultivated overnight on agar plates with selection antibiotics. Single monocolony was transferred to 100 µl of ddH_2O using a toothpick. Bacteria in ddH_2O were lysed by incubation in 96°C for 5 minutes. From this lysate, 2.5 µl was used as template. Triton X-100 1% solution (2 µl) was added in every sample. The rest of PCR mixture and PCR program were performed as described in chapter 3.2.3.9.

3.2.3.10. Sequencing

Plasmid to be sequenced was isolated from bacteria cultivated overnight (see chapter 3.2.3.1.). Sequencing mix was prepared according to instructions from sequencing laboratory. It contained 600 – 1200 ng (100 ng of DNA ~ 100 bp to be tested) of purified plasmid, 3.2 pmol of primer and ddH_2O. The total volume was 14 µl.

The sequencing reaction was performed by the Laboratory of DNA sequencing at the Faculty of Natural Sciences in Charles University. The laboratory uses two sequencing analysers - 3130 Genetic Analyzer and 3130xl Genetic Analyzer (Applied Biosystems).

3.2.4. Work with bacteria

3.2.4.1. Cultivation

Bacteria were cultivated either in a liquid medium (in Erlenmayer flask) or on agar plates. Selective antibiotics were added to medium prior to inoculation of bacteria. Bacteria were cultivated overnight (12 – 16 hours) at 37°C in a shaker (liquid medium) or an incubator (agar plates).

3.2.4.2. Storage

Short-term storage (up to one month) of bacteria cultivated on agar plates took place at 4°C. Long-term storage of bacteria cultivated in liquid medium took place at -80°C in form of conserves. Samples of bacteria cultivated overnight were five times concentrated and stored in 20% glycerol.

3.2.4.3. Competent cells preparation

Medium TPN was prepared and sterilized by autoclaving one day prior to preparation. Bacteria XL-1 blue or BL-21(DE3) were inoculated in 10 ml of TPN medium and cultivated overnight. Next morning, bacteria were diluted in TPN medium to OD_{600} = 0.1, bacteria grew until OD_{600} reached ~0.6. Bacteria were centrifuged in a refrigerated centrifuge (4000 g, 10 min), and then washed twice in cold sterile ddH_2O (300 ml and 150 ml) and then twice in cold sterile 10% glycerol (16 ml and 10 ml) and resuspended in 1 ml of cold sterile 10% glycerol. Final suspension was distributed to microtubes (50 µl or 100 µl aliquots), frozen in liquid nitrogen and stored at -80°C.

3.2.4.4. Cell transformation by electroporation

Stock of competent bacteria was thawed on ice. Bacterial suspension, 50 µl, was mixed with 1.5 µl of ligation mixture (or negative control) or plasmid in an electroporation cuvette (interelectrode distance: 2 mm). Electroporation pulse (capacitance: 25 µF, voltage: 2.5 kV, resistance: 200 Ω) was applied for 4.5 – 5 milliseconds. Bacterial suspension was diluted in 1 ml of SOC medium immediately afterwards and incubated in Erlenmayer flask at 37°C in a shaker for one hour. Bacteria were inoculated on agar plates with selective antibiotics in various dilutions and incubated overnight at 37°C.

3.2.4.5. Protein expression (standard protocol)

Bacteria BL-21 were used for protein expression. BL-21 bacteria contain T7 RNA polymerase gene in araB locus. Expression of the polymerase can be induced by treatment with IPTG, which leads to transcription and following translation of genes encoded under T7 RNA promoter.

Bacteria were inoculated in 10 ml of culture medium with selective antibiotics and cultivated overnight at 37°C in a shaker. In the morning, bacterial suspension was diluted in culture medium to OD_{600} = 0.1 and cultivated until OD_{600} reached ~0.7. Then, protein expression was induced by adding IPTG to final concentration 0.1 mM (sample bacteria), some bacteria were cultivated without IPTG and served as non-induced controls. Bacteria were cultivated at 28°C for 2 - 3 hours and then OD_{600} was measured once again in sample and control bacteria to monitor bacterial growth ability. From sample and control bacteria, 1.5 ml aliquots were centrifuged in a refrigerated centrifuge (4000g, 10 min). Dry pellets were stored at -20°C or immediately used for lysates preparation.

3.2.4.6. Optimization of expression

Optimization of expression is often necessary to obtain sufficient yield of the expressed protein. At first, several conditions were optimized using the standard protocol (3.2.4.6.1. and 3.2.4.6.2.). Later, an alternative protocol was also applied (3.2.4.6.3.).

3.2.4.6.1. Cultivation temperature

Bacteria were prepared and cultivated as described above until OD_{600} reached ~0.7. Protein expression was induced by adding IPTG to final concentration 0.1 mM. Then, bacteria were cultivated at 15°C, 20°C or 28°C overnight in a shaker. In the morning, bacteria were centrifuged and lysed as described above.

3.2.4.6.2. IPTG concentration

Bacteria were prepared and cultivated as described above until OD_{600} reached ~0.7. Protein expression was induced by adding IPTG to final concentration 1mM (the highest concentration commonly used for inducing expression). Bacteria were cultivated at 28°C and the above described procedure was applied. Such high concentration of IPTG exhibited a toxic effect on bacteria as they multiplied much more slowly compared to non-induced control (based on comparison of OD_{600} at the end of expression).

3.2.4.6.3. Alternative protocol

Bacteria were inoculated in 10 ml of culture medium with selective antibiotics in the morning. Samples were cultivated at 37°C for about 8 hours. Bacterial suspension was diluted in culture medium to OD_{600} = 0.1 and protein expression was induced by addition of IPTG to final concentration 0.1 mM. Bacteria were cultivated overnight at 28°C. In the morning bacteria were centrifuged and lysed as described in the standard protocol.

3.2.5. Work with proteins

3.2.5.1. Cell lysis (Laemmli buffer) – bacteria

Lysis in Laemmli buffer or "cell lysis" was applied to reveal PBS-soluble and insoluble proteins from bacterial samples. Dry pellet of bacteria was resuspended in small amount of PBS (usually 150 µl for 1.5 ml aliquot of initial bacterial suspension) and 2/3 of volume of 5x Laemmli buffer (resulting in 2x Laemmli buffer). Samples were stirred well on vortex and incubated at 100°C for 10 minutes. After boiling, samples were centrifuged in a refrigerated centrifuge (20000 g, 10 min). Supernatant was tested on dot blot, western blot or SDS PAGE.

3.2.5.2. Native lysis (liquid nitrogen) – bacteria

Lysis in liquid nitrogen or "native lysis" was applied to reveal PBS-soluble proteins from bacterial samples. Dry pellets of bacteria were resuspended in a small amount of PBS (usually 250 µl for 1.5 ml aliquot of initial bacterial suspension) with protease inhibitor cocktail. Samples were frozen in liquid nitrogen and left to thaw repeatedly (three times). Thawed samples were centrifuged in a refrigerated centrifuge (20000 g, 10 min). Supernatant was tested on dot blot, Western blot or SDS PAGE.

3.2.5.3. Lysis in RIPA buffer – Sf9

Cells (Sf9 cell line) infected with a baculovirus or mock infected were collected 72 h.p.i., centrifuged (90 g, 10 min) and washed with PBS. Collected cells were resuspended in a small volume of RIPA buffer (usually 250 µl per a Ø 10 cm well) with protease inhibitor cocktail. Samples were stirred repeatedly on vortex and incubated on ice for 20 minutes. Samples were centrifuged in a refrigerated centrifuge (20000 g, 10 min). Supernatant was tested on dot blot, Western blot or SDS PAGE.

3.2.5.4. Dot blot

Protein samples were applied in drops on a nitrocellulose membrane (volume range 0.5 – 3 µl). Detection of protein was immunogenic (see chapter 3.2.5.6.).

3.2.5.5. SDS PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) is a method commonly used to separate denatured proteins exclusively by their molecular weight.

3.2.5.5.1. Electrophoresis

Apparatus was assembled according to manufacturer's instructions, correct assembly was verified by filling the apparatus with ddH_2O. Glasses were dried properly and separation gel was poured up to a level marked earlier (about 1 cm below the end of comb). Separation gel was overlaid with ddH_2O and polymerized for 30 minutes. Afterwards, ddH_2O was dried properly and stacking gel was poured in the apparatus and the comb was put on top. Gel polymerized for 30 minutes. Polymerized gel was transferred into an apparatus for electrophoresis and covered with running buffer. Sample holes were washed properly with the washing buffer and samples and molecular weight marker were applied (5 – 20 µl). Protein samples migrated through the stacking gel for 30 minutes at 80 V and through the separation gel at 140 V until bromphenol blue marker reached the bottom of the gel.

3.2.5.5.2. Gel staining

Gel was fixed in a fixing solution (with methanol and $HClO_4$, see chapter 3.1.14. for contents) and incubated in a CBBG solution overnight. Afterwards, the gel was incubated in dH_2O for several days to remove excess colour.

3.2.5.5.3. Western blot

After electrophoresis, gel was incubated in blotting buffer for 10 minutes and was incorporated into a blotting sandwich. The blotting sandwich was assembled as follows – blotting pad, filter papers, Whatman paper, gel, nitrocellulose membrane, Whatman paper, filtration papers and blotting pad. All components were soaked in blotting buffer prior to incorporation into the sandwich. Blotting sandwich was installed into a blotting apparatus, proteins were transferred at 250 mA for 3 hours. Detection of protein was immunogenic (see chapter 3.2.5.6.).

3.2.5.6. Immunodetection of proteins

Membrane was blocked for at least 30 minutes in blocking solution (5% low-fat milk in PBS). Primary antibody diluted in the blocking solution was applied on the membrane and incubated for at least one hour. Membrane was washed three times in PBS (10 minutes each washing). Secondary antibody conjugated with horse radish peroxidase (HRP) diluted in the blocking solution was applied on the membrane and incubated for 30 minutes. Membrane was washed three times in PBS (10 minutes

each washing). A luminol solution was applied on membrane for 30 seconds in a darkroom. Medical Xray film blue (Agfa) was exposed to membrane signal (to detect subsequent light emitting reaction) for 30 seconds – 15 minutes. The film was incubated in a developer and a fixer, washed in distilled water and dried.

3.2.5.7. Protein purification on HIS-Select® Nickel Affinity Gel

Purification of recombinant protein expressed in bacteria was performed using HIS-Select® Nickel Affinity Gel according to manufacturer's instructions. Expression of recombinant protein was induced in bacteria for several hours, bacteria were collected and lysed by native lysis (see chapter 3.2.5.2.). 50 µl of HIS-Select® Nickel Affinity Gel suspension was first washed with equilibration buffer (50mM sodium phosphate and 0.3M sodium chloride, pH 8) and centrifuged (5000 g, 30 seconds). Supernatant was discarded and 100 µl of bacterial lysate was added and incubated for one hour on a shaker. Suspension was centrifuged and supernatant was collected and labelled as "flow through". HIS-Select® Nickel Affinity Gel suspension was washed twice with 500 µl of wash buffer (50mM sodium phosphate, 0.3M sodium chloride, pH 8), centrifuged and supernatant was collected and labelled as "wash". HIS-Select® Nickel Affinity Gel was resuspended in 50 µl of elution buffer (50mM sodium phosphate, 0.3M sodium chloride, 250mM imidazole, pH8), centrifuged, respuspended in 50 µl of elution buffer once more and centrifuged once more. Both supernatants were combined and labelled as "elute". Pellet including HIS-Select® Nickel Affinity Gel was labelled as "gel". Collected samples were examined by SDS PAGE and Western blot.

3.2.6. VLPs isolation

Virus-like particles (VLPs) produced by recombinant baculovirus (see chapter 3.2.7.) were isolated from 20 Ø 10 wells of Sf9 cells. Cells were infected with recombinant baculovirus and collected 4 d.p.i. They were centrifuged, washed with PBS and stored at -20°C (dry pellets).

3.2.6.1. Sucrose cushion ultracentrifugation

Pellets were resuspended in several ml of B buffer and under-layered with 10% sucrose solution. Ultracentrifugation in SW28 rotor at 25,000 rpm ran for three hours. Supernatant was removed and pellets slowly dissolved in B buffer.

3.2.6.2. Isopycnic ultracentrifugation in CsCl gradient

Dissolved pellets were homogenized in Potter homogenizer repeatedly (6 x 10 plunges) on ice. Perfectly homogenized suspension was transferred to an ultracentrifugation tube and B buffer was added until the weight of suspension reached 7.9 g. Caesium chloride (3.79 g) was added to

suspension to obtain density used for polyomaviral VLPs separation. Correct density was verified by measuring refraction index. Isopycnic centrifugation was performed in SW41 rotor at 35,000 rpm for 24 hours. Settled gradients (formed during ultracentrifugation) were separated into fractions using peristaltic pump. The presence of protein VP1 in fractions was tested by dot blot and based on the results, fractions were combined into two aliquots.

3.2.6.3. Dialysis

The obtained aliquots contained high concentration of CsCl which had to be removed by dialysis. Dialysis membranes were prepared by incubation in boiling dH_2O for 10 minutes. Membranes were filled with virus suspension and closed by dialysis clips. Dialysis was performed in 2 litres of B buffer at 4°C and constant stirring. After 2 hours, B buffer was replaced with fresh dose, dialysis continued overnight. Another sucrose cushion centrifugation was applied to concentrate and purify viral samples. Final samples were stored at -70°C.

3.2.6.4. Hemmaglutination test

Hemagglutination test can be applied on some polyomaviral particles thanks to ability of major structure protein VP1 to bind sialic acid and cause agglutination of erythrocytes. Hemagglutination test is an easy way to determine the concentration of particles in HAU (hemagglutination units).

Guinea pig blood was processed to obtain a 0.4% suspension of erythrocytes in PBS by Ivana Polívková (technician). Viral suspension was diluted in BSA in PBS solution by binary dilution, which produced dilution 10^{-1} – 10^{-12}. From each dilution, 50 µl was mixed with 50 µl of BSA in PBS solution and 50 µl of erythrocyte suspension in a 96-well plate. The plate was incubated at 4°C for several hours and hemagglutination titer was determined using a formula empirically determined for MPyV capsids:

$$HAU \cdot 200 \cdot 10^7 = particles/ml$$

HAU is an inverse value of dilution of 50 µl viral aliquot (with 5 µl of virus suspension), “200” represents the conversion from 5 µl of viral suspension to 1 ml, “10^7” represents amount of particles present in 1 HAU. This concentration calculation was designed to determine concentration of MPyV capsids.

3.2.6.5. Bradford method

Another method to establish concentration of VLPs suspension is the determination of protein concentration. The Bradford method is based on colour change accompanying reaction of CBBG

and basic and aromatic amino acids. This colour change is measured by changes in absorbance at 595 nm (A_{595}).

Calibration standards (BSA in B buffer) were prepared in following concentration: 0, 0.25, 0.5, 0.75, 1 mg/ml. Calibration standards were mixed with 1 ml of Bradford reagent, mixed properly and A_{595} was measured. A regression equation was plotted based on the results. Viral suspension was diluted in B buffer, mixed with 1 ml of Bradford reagent and A_{595} was measured. Protein concentration in samples was determined based on the regression equation.

3.2.6.6. Electron microscopy

All electron microscopy samples were prepared and photographed by Mgr. Martin Fraiberk.

3.2.6.6.1. Negative staining

Copper gauze was put on top of 5 µl drop of viral suspension and incubated for 10 minutes. Gauze was washed twice on top of 100 µl drop of ddH_2O (30 seconds each washing) and twice stained on top of 100 µl drop of 2% phosphowolframic acid (1 minute each incubation). Excess stain was removed by soaking with filter paper and the gauze was dried.

3.2.7. Recombinant baculovirus preparation

3.2.7.1. Bac-to-bac system

Baculoviral system is an efficient expression system with high protein yields. It has been proven as suitable for production of VLPs, especially those derived from polyomaviruses and papillomaviruses. Recombinant baculovirus encodes the gene of interest under polyhedrin (PH) promoter. Polyhedrin promoter is active in late phases of baculoviral infection producing very high yields. Another promoter used for the gene of interest expression is p10 promoter, p10 is a late protein produced in high yields as well.

Bac-to-bac system from Invitrogen was used to prepare recombinant baculovirus. Bac-to-bac system is an efficient and fast system based on site-specific transposition for generating recombinant baculoviruses. The whole procedure was performed according to manufacturer's instructions.

Initially a recombinant plasmid was generated. Plasmid pFastBac Dual was used due to its capacity to contain two inserts of interest. DNA for insertion was generated by PCR and inserted by ligation. The recombinant plasmid contains a cassette for site-specific transposition, this cassette contains the two

inserts (under control of PH and p10 promoters) and a gentamicin resistance gene. Final recombinant plasmid was verified by sequencing and electroporated into DH10Bac bacteria.

DH10Bac bacteria contain a bacmid with identical transposition site to those in the recombinant plasmid. After electroporation, site specific transposition between plasmid and bacmid occurs, resulting in bacmid (baculoviral genome) with inserts of interest under PH and p10 promoters. A marker of successful transposition is disruption of gene for β-galactosidase. Bacterial colonies, where transposition was successful, cannot convert X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside present in agar) to blue product and they appear white.

Several white colonies were inoculated in medium with selective antibiotics (gentamicin, kanamycin, tetracyclin) and cultivated overnight. Bacmids were isolated from colonies and insert presence was verified by PCR and sequencing of PCR products. Verified bacmids were transfected into Sf9 cells.

Supernatant from cells was collected 72 h.p.i., purified by plaque assay and used as viral inoculum for repeated infections. Transfected and infected cells were lysed and checked on dot blot and Western blot.

3.2.7.2. Plaque assay

Cells (Sf9 cell line) were passaged to 10 plates (Ø 6 cm, $2 \cdot 10^6$ cells per well) one hour prior to infection. Baculoviral inoculum was diluted 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}; 0.5 ml of diluted inoculum was used for cells infection (see chapter 3.2.1.3. for the details of the infection procedure). Infection was performed in duplicates and mock infected cells served as negative control.

Infection took one hour and then cells were covered with agarose medium (1.5% SeaPlaque agarose, 0.5x insect cells medium, 10% FBS, antibiotics mix). Plates were incubated in sterile conditions until agarose medium gelled and then incubated in incubator. After 10 days, visible plaques were counted and viral concentration was determined using formula:

$$plaque\ amount \cdot 10^6 \cdot 2 = pfu/ml$$

4. Results

4.1. Preparation of monoclonal antibody against unique part of VP2 protein from BK virus (BKV)

Our laboratory research is focused on polyomaviruses, their life cycle and interactions with a host cell. BK virus (BKV) is a member of the *Polyomaviridae* family and can cause severe disorders in humans. Intention to study interactions of this important pathogen with host cell raises needs for antibodies specific to viral antigens. BKV genome encodes six proteins, three of which are structural proteins. Major structural protein VP1 forms pentamers, the structural subunits of BKV capsid. Minor structural proteins VP2 and VP3 are closed in cavity of VP1 pentamers and are not exposed on intact virion surface. Besides their structural role in virus particle, minor proteins are assumed to have also other important roles during infection. These assumptions are based on observations made on model polyomaviruses MPyV and SV40, for BKV it is largely unknown. Sequence of minor proteins VP2 and VP3 is identical in major part of the protein, while VP3 is a shorter version of VP2. In other words, VP2 has additionally a unique sequence on N-terminus (Fig. 4.1). An antibody against this unique part, in combination with an antibody against the identical part would allow us to examine differences in localisation of the two minor proteins.

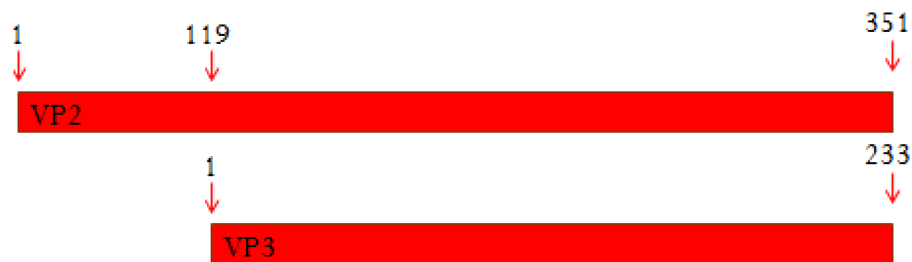


Fig.4.1: Scheme of protein sequences of BKV minor proteins VP2 and VP3. Numbers represent amino acids. VP2 sequence includes a 119-amino acid long unique part.

4.1.1. Mouse immunisation and serum testing

Expression and purification of antigen was carried out by Martina Verdánová (M. Verdánová, diploma thesis, 2011). As antigen for immunisation we prepared a fusion protein, where the unique part of protein VP2 was fused with His-tag and S-tag (VP2 unique). His-tag was used in affinity purification of the protein. S-tag is part of the multiple-cloning site of used vector (pET29b) and avoiding its presence would require multiple cloning steps, while its presence should not (and did not) interfere with expression, purification or immunisation. This construct was expressed in bacteria and purified on HisTrap FF crude column (GE Healthcare). Concentration was 0.23 mg/ml measured by the Bradford method.

Two BALB/c mice were immunised intraperitoneally with 25 µg of antigen dissolved in 100 µl PBS resuspended in 100 µl of complete Freund´s adjuvant (CFA). Immunisation was repeated twice (after 2 and 4 weeks). Incomplete Freund´s adjuvant was used for booster dose. A blood sample from *vena caudalis* was taken from mice six weeks after the first immunisation.

Blood serum was obtained by sample centrifugation and presence of specific antibody was tested by immune dot blot. Results confirmed the presence of antibodies against VP2 in mouse blood (Fig. 4.2). Both sera were shown to contain specific anti VP2 antibodies. While immune-reaction of mouse no. 2 was stronger, this mouse was chosen for hybridoma preparation.

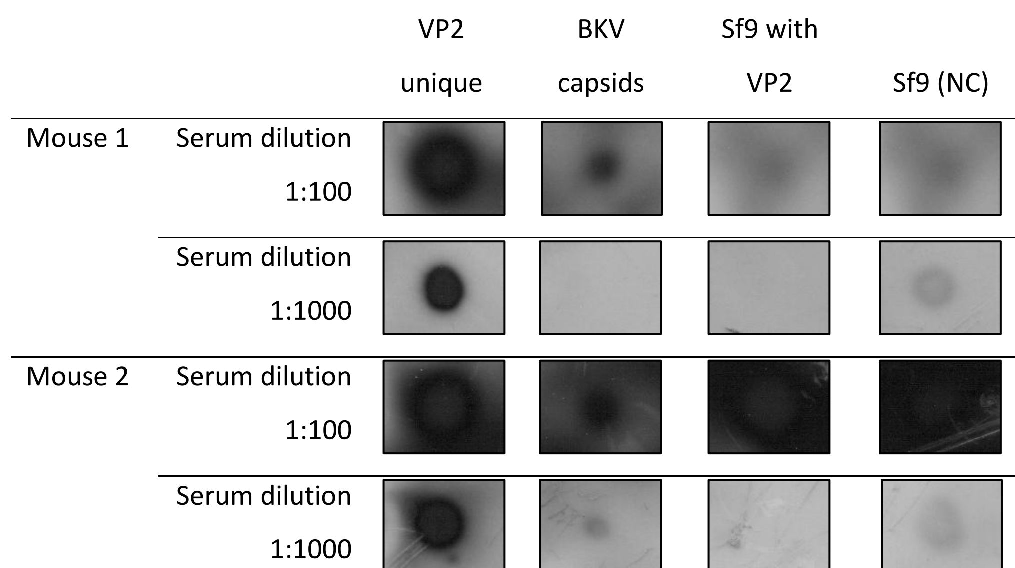


Fig. 4.2: Sera obtained from both immunized mice contain antibodies recognizing BKV VP2 protein. First column: The antigen used for immunisation (VP2 unique, recombinant protein). Second column: Fraction of empty viral capsids from isolation of BK virus (BKV capsids consisting of VP1, VP2 and VP3 native proteins, provided by Violeta Bakardijeva). Third column: RIPA lysates of Sf9 cell (see chapter 3.2.5.3. for details of the procedure) infected with a recombinant baculovirus producing VP2 protein of BKV. Fourth column: RIPA lysate of mock-infected Sf9 cells (Sf9(NC)). Sera from immunized mice were used as primary antibodies, two serum dilutions (in blocking solution) were used, 1:100 and 1:1000. Secondary antibody: goat anti-mouse antibody conjugated with horse radish peroxidase (HRP) (dilution 1:1000). Film was exposed for 30 seconds.

4.1.2. Hybridoma preparation

Mouse was sacrificed and its spleen (Fig. 4.3) was removed under sterile conditions. Spleen cells were fused with myeloma cells Sp2/0 (see chapter 3.2.2.2. for details of the procedure). Fused cells were distributed into five 96-well plates on a feeder cell layer. Three days after the fusion, cells were treated with HAT medium with geneticin (concentration 200 ng/µl) for three days. After that cells

were cultivated in complete HAT media (see chapter 3.1.11.2. for contents). Culture media of hybridomas were tested for the presence of specific antibodies.

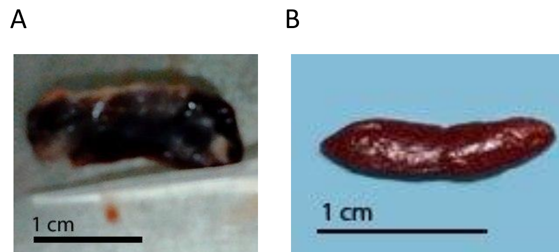


Fig. 4.3: Comparison of spleen from immunised (A) and non-immunised (B) mice. Spleen from immunised mouse is visibly enlarged due to ongoing immune reaction. Black line represents 1 cm, immunised spleen is about 0.6 cm bigger than the non-immunised one. (B) was adapted from *http://amrita.vlab.co.in/?sub=3&brch=70&sim=200&cnt=1*.

4.1.3. Hybridoma testing

Hybridoma culture media were tested mainly by FACS analysis (Fig. 4.4). Insect cells (Sf9 line) infected with recombinant baculovirus expressing VP2 fused with His-tag and baculovirus expressing VP1 and VP2 proteins of BKV were used as antigens (see chapter 3.2.2.4.1. for details of the procedure). FACS analysis revealed over 130 positive clones, five of them were further cloned, the rest of hybridomas were stored in liquid nitrogen.

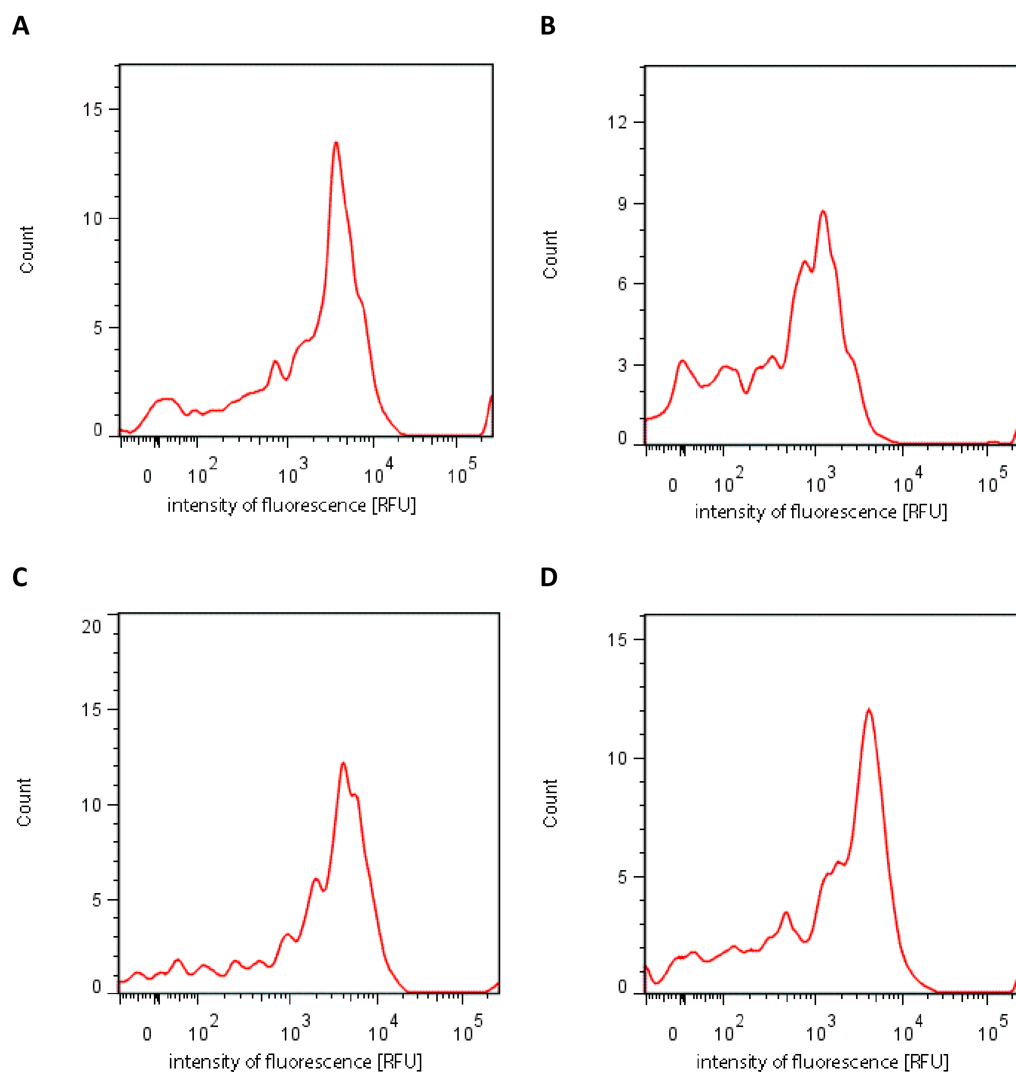


Fig. 4.4: **Hybridoma clones producing antibody of desired specifity were tested by FACS analysis.** FACS analysis revealed over 130 positive clones. Sf9 cells infected with baculovirus producing VP1 and VP2 proteins of BKV were used and stained with: **A**: anti His-tag antibody (positive control), **B**: HAT medium (negative control), **C** and **D**: hybridoma culture medium (from clones 3-C8 and 4-B2, respectively). Hybridomas strongly producing antibodies with highest affinity to VP2 unique were further cloned. Intensity of fluorescence (X axis) is shown in RFU (relative fluorescence units), count (Y axis) represents number of cells

4.1.4. Hybridoma cloning

Hybridomas identified as antibody producers were cloned in order to obtain purified monocolonies with stabilized production of monoclonal antibodies. Hybridoma cells from one well were distributed to a 96-well plate on a feeder cell layer using limited dilution method (see chapter 3.2.2.3.2. for details of the procedure).

4.1.5. Antibody collection

Due to low stability of specific antibodies producing hybridoma cell lines, we started collecting culture media from all positive wells. Various clones produced various VP2 unique-specific antibodies and so the collected yield resembled a polyclonal antibody. Collected media were tested by dot blot (Fig. 4.5) and by FACS analysis (Fig. 4.6). Test showed that collected polyclonal antibody is unspecific, or rather His-tag-specific.

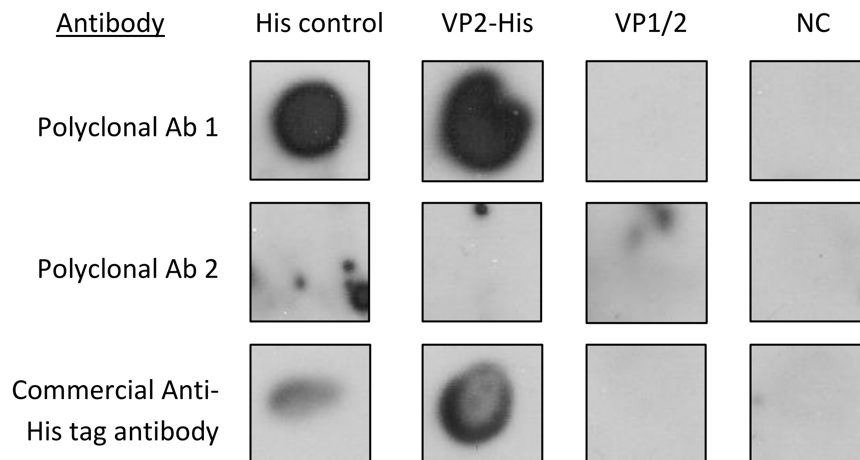
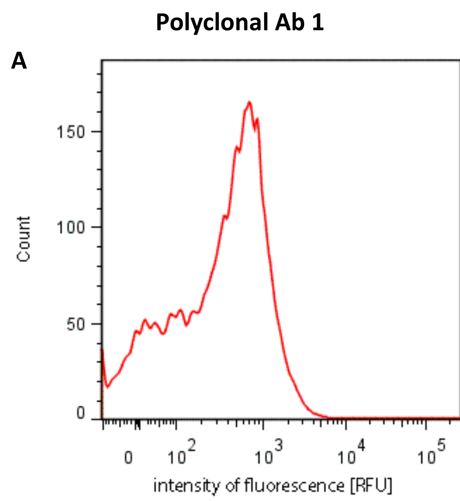


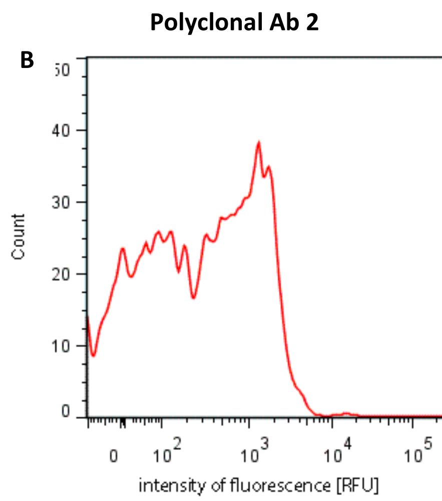
Fig.4.5: Immuno-dot blot testing of collected polyclonal antibody. First column: VP1 protein of MPyV fused with CAP and His-tag was used as positive control (His control, kindly provided by M. Fraiberk) Second column: Lysate of Sf9 cells infected with VP2 protein of BKV fused with His-tag producing baculovirus. Third column: Lysate of Sf9 cells infected with baculovirus producing VP1 and VP2 proteins of BKV. Fourth column: Lysate (RIPA) of mock infected Sf9 cells. Polyclonal collection 2 was highly unspecific, Polyclonal collection 1 was rather His-tag-specific. Commercial antibody against His-tag (dilution 1:100) was used as control.

Polyclonal Ab 1 **Polyclonal Ab 2**

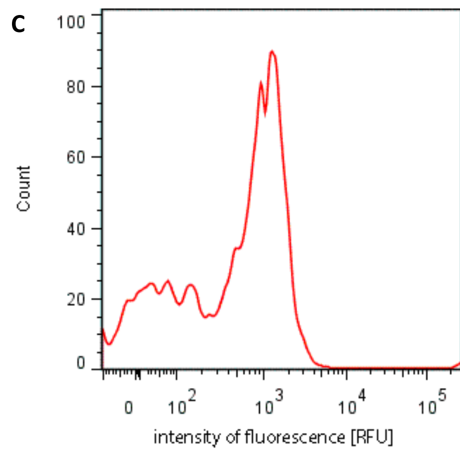
A



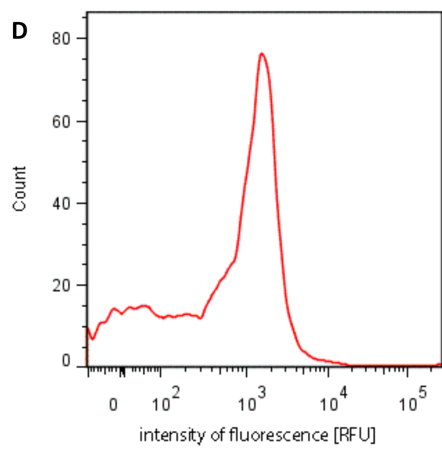
B



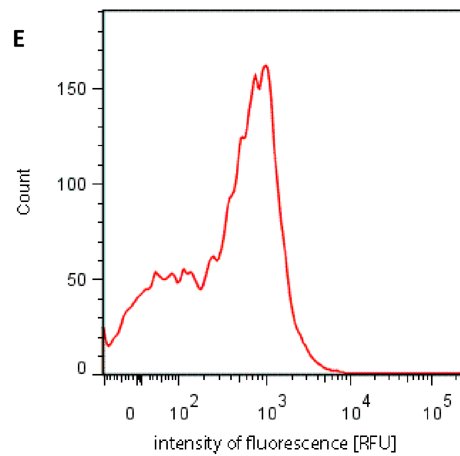
C



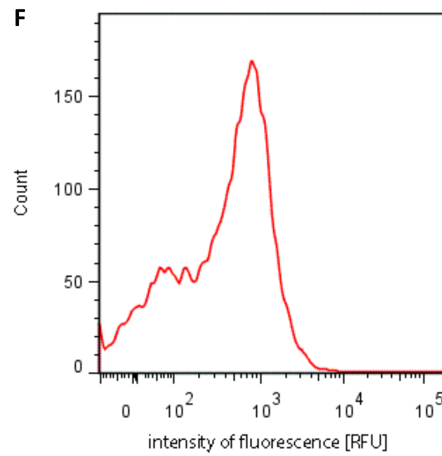
D



E



F



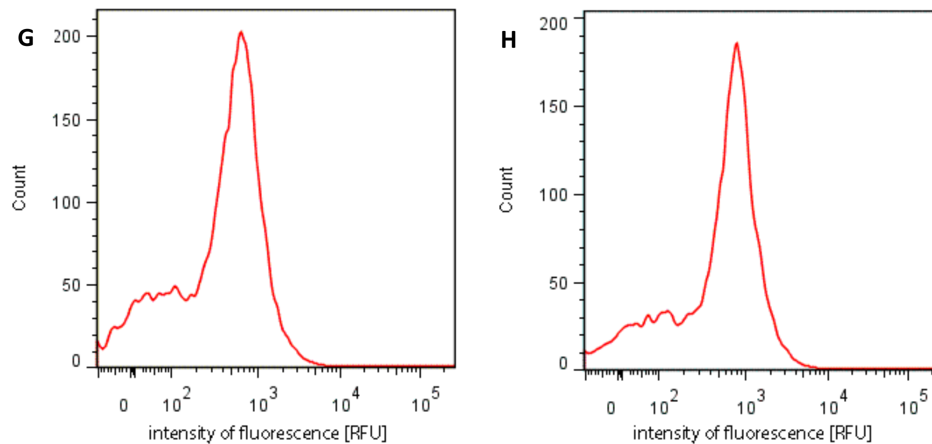


Fig. 4.6: Interaction of collected PAb with antigens. Intensity of Ab-antigen binding was tested by immunostaining and measured by FACS. Sf9 cells were used as antigens: **A**,**B**: infected with recombinant baculovirus producing VP2 protein of BKV fused with His-tag; **C**,**D**: infected with recombinant baculovirus producing VP1 and VP2 proteins of BKV; **E**,**F**: wild type baculovirus; **G**,**H**: mock infected cells. Antigens were stained with the collected polyclonal antibodies (first column polyclonal collection 1, second column polyclonal collection 2). Intensity of fluorescence (X axis) is shown in RFU (relative fluorescence units), count (Y axis) represents number of cells

4.1.6. Samples thawing and maintenance

Over fifty clones were thawed, but often succumbed to prokaryotic or fungal contamination before testing. Tested samples were either negative or died out shortly after testing.

4.2. Preparation of monoclonal antibody against Merkel Cell Polyomavirus (MCPyV) VP2 protein

Merkel Cell Polyomavirus (MCPyV) is a newly discovered member of the *Polyomaviridae* family. It is connected to majority of (if not all) cases of Merkell Cell Carcinoma, an aggressive form of human skin cancer. MCPyV was described only five years ago. Research on the virus is complicated by the fact that permissive cell line still has not been found. It is thereby impossible to propagate and purify the virus by standard procedures. Despite these difficulties, our laboratory conducts research on interactions of MCPyV proteins with cell structures. To localise viral proteins in cell, we prepare monoclonal antibodies – against VP1 (P. Sauerová, diploma thesis, 2013) and against VP2.

4.2.1. Mice immunisation and serum testing

Preparation of antigen, mice immunisation and serum testing was carried out by Mgr. Martina Kojzarová.

Two BALB/c mice were immunised twice by the gene gun method with modified ph2m plasmid (see chapter 3.2.2.1.2.) and boosted by lysates of 3T3 cells (transfected with the same plasmid) with intraperitoneal injection. After that a blood sample from *vena caudalis* was taken and tested.

Blood sample was centrifuged and serum was tested by immuno-dot blot to prove the presence of antibodies specific to VP2 protein of MCPyV (Fig.4.7). The serum testing confirmed that mouse developed antibodies against ph2m transfected 3T3 lysates (used for immunisation) and probably also against VP2 protein of MCPyV (as tested by staining of lysate of Sf9 cells infected with recombinant baculovirus produding proteins VP1 and VP2 of MCPyV,this lysate had not been used for immunisation). Unimmunised mouse did not posses antibodies against 3T3 lysate with VP2 protein, which suggests these antibodies do not occur naturally. Three days before cell fusion, antibody production was boosted by additional injection of 3T3 lysate with VP2 protein.

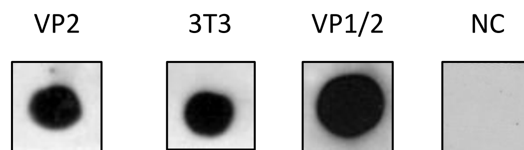


Fig.4.7: Serum from immunized mice recognized MCPyV VP2 protein. 3T3 cells were transfected with a ph2m plasmid producing VP2 protein of MCPyV (**VP2**) or were mock-transfected (**3T3**). Sf9 cells were infected with a recombinant baculovirus producing VP1 and VP2 proteins of MCPyV (**VP1/2**) and were used as antigens for testing. Cells were lysed in RIPA buffer (see chapter 3.2.5.3. for details of the procedure). Lysate of 3T3 cells transfected with ph2m plasmid stained with blood serum of a non-immunized mouse was used as negative control (**NC**). Goat antibody against mouse immunoglobulins fused with horse radish peroxidise was used as secondary antibody (dilution 1:1000). Experiment was performed by M. Kojzarová.

4.2.2. Hybridoma preparation

Mouse spleen cells were fused with myeloma cells Sp2/0 (see chapter 3.2.2.2. for details of the procedure). Fused cells were distributed into five 96-well plates on a feeder cell layer. Three days after the fusion, cells were treated with HAT medium supplemented with geneticin (concentration 200 ng/µl). Geneticin was presented through three days, after that it was replaced by HAT medium (see chapter 3.1.11.2. for contents). Culture media of hybridomas were tested for the presence of specific antibodies.

4.2.3. Hybridoma testing

Hybridoma culture media were tested by FACS analysis (Fig. 4.8). Insect cells (Sf9 line) were infected with recombinant baculovirus expressing VP1 and VP2 proteins of MCPyV (see chapter 3.2.2.4.1. for details of the procedure). FACS analysis revealed 38 positive clones after fusion (in the first

generation). The most positive clones (from each generation) were further cloned and tested, the rest was stored in liquid nitrogen.

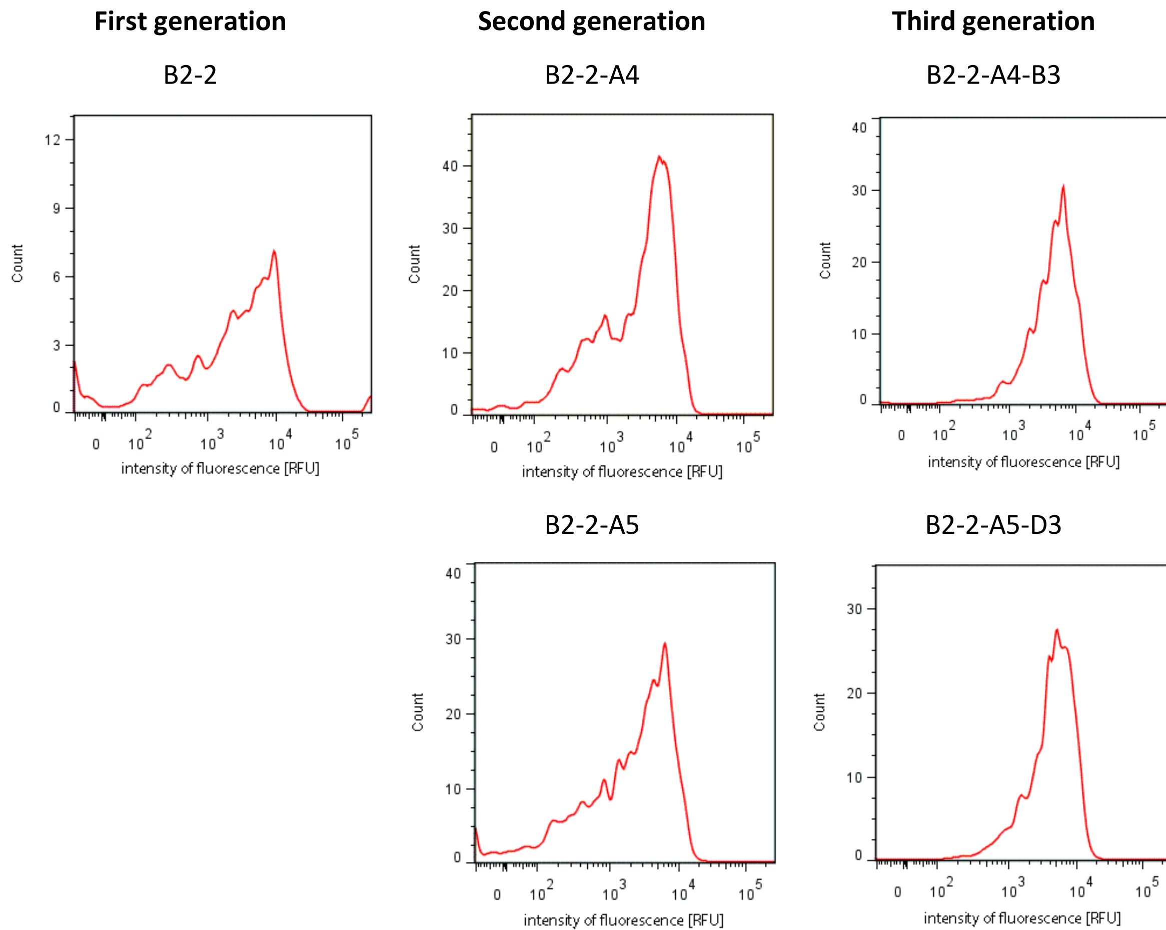


Fig. 4.8: **Repeatedly cloned hybridomas recognized their antigen with higher specificity.** Intensity of fluorescence was measured by FACS analysis. Sf9 cells infected with baculovirus producing VP1 and VP2 proteins of MCPyV were used and stained with hybridoma culture media. Shown is the lineage giving rise to 9 clones grown and tested in larger scale (see chapter 4.2.5.) Intensity of fluorescence (X axis) is shown in RFU (relative fluorescence units), count (Y axis) represents number of cells

4.2.4. Hybridoma cloning

Positive hybridoma colonies were cloned in order to obtain purified monocolonies (producing monoclonal antibody). Hybridomas producing antibody of desired specifity from one well were redistributed to 96 wells (one 96-well plate) on a feeder cell layer by limited dilution method (see chapter 3.2.2.3.2. for details of the procedure).

In the third generation (after third round of cloning), bacterial contamination appeared in hybridoma cultures. An attempt to thaw some frozen aliquots instead was made, but the contamination reappeared in these aliquots as well. Profiting from resistance to geneticin (possessed by myelomas

used for fusion), cultures were treated with 500 ng/ µl of geneticin from that moment on. Bacterial contamination diminished gradually and in most samples finally disappeared. Antibody production seemed not to be affected by this treatment as checked by FACS analysis (data not shown).

In the fourth generation of hybridomas, several clones were marked as established producing clones based on FACS analysis results (Fig.4.9). From these clones, nine were chosen for cultivation in larger scale and further testing.

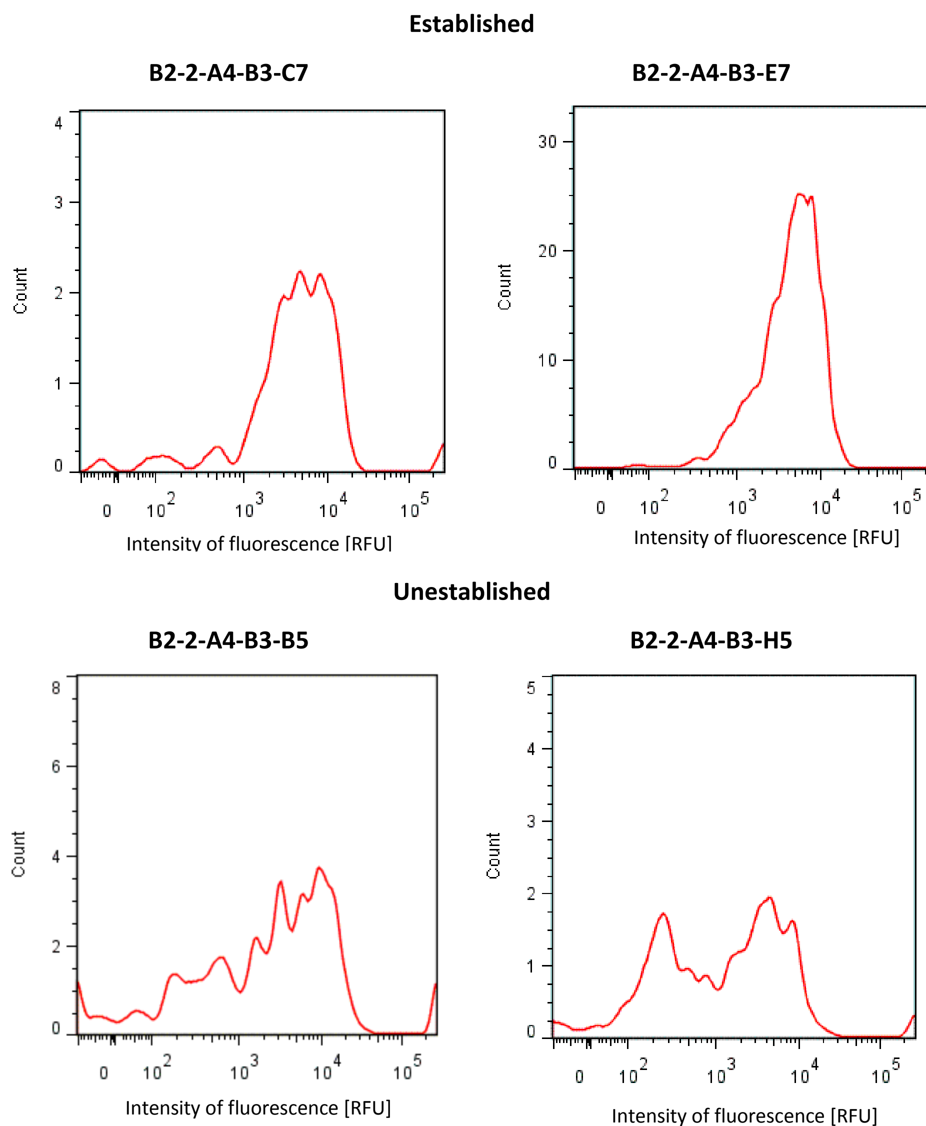


Fig.4.9: FACS analysis of culture media of selected hybridomas (fourth generation). Sf9 cells infected with baculovirus producing VP1 and VP2 proteins of MCPyV were used as antigens and stained with culture media (as primary antibody). Results representing regular distribution of positively stained cells (established, clones B2-2-A4-B3-C7 and B2-2-A4-B3-E7) indicate established producing clones. Results with irregular distribution (unestablished, clones B2-2-A4-B3-B5 and B2-2-A4-B3-H5) imply rather a heterogeneous population or low antibody productivity. Intensity of fluorescence (X axis) is shown in RFU (relative fluorescence units), count (Y axis) represents number of cells

4.2.5. Testing and cultivation in larger scale

Nine clones, labelled B-B7, B-C7, B-E7, B-F6, B-F7, B-G6, B-G7, C-B7, C-D7 (B and C correspond to plates from fourth round of cloning, originating from clones B2-2-A4-B3 and B2-2-A5-D3, respectively). Each clone was transferred to one well in a 24-well plate, later to a 6-well plate and finally to a 25 cm^2 cultivation flask. Culture medium was collected during passages or when replaced with fresh one. Media from the nine clones were tested by dot blot using native antigens, dot blot using denatured antigens, FACS analysis and some by immunofluorescent staining as well (Fig. 4.10A-J).

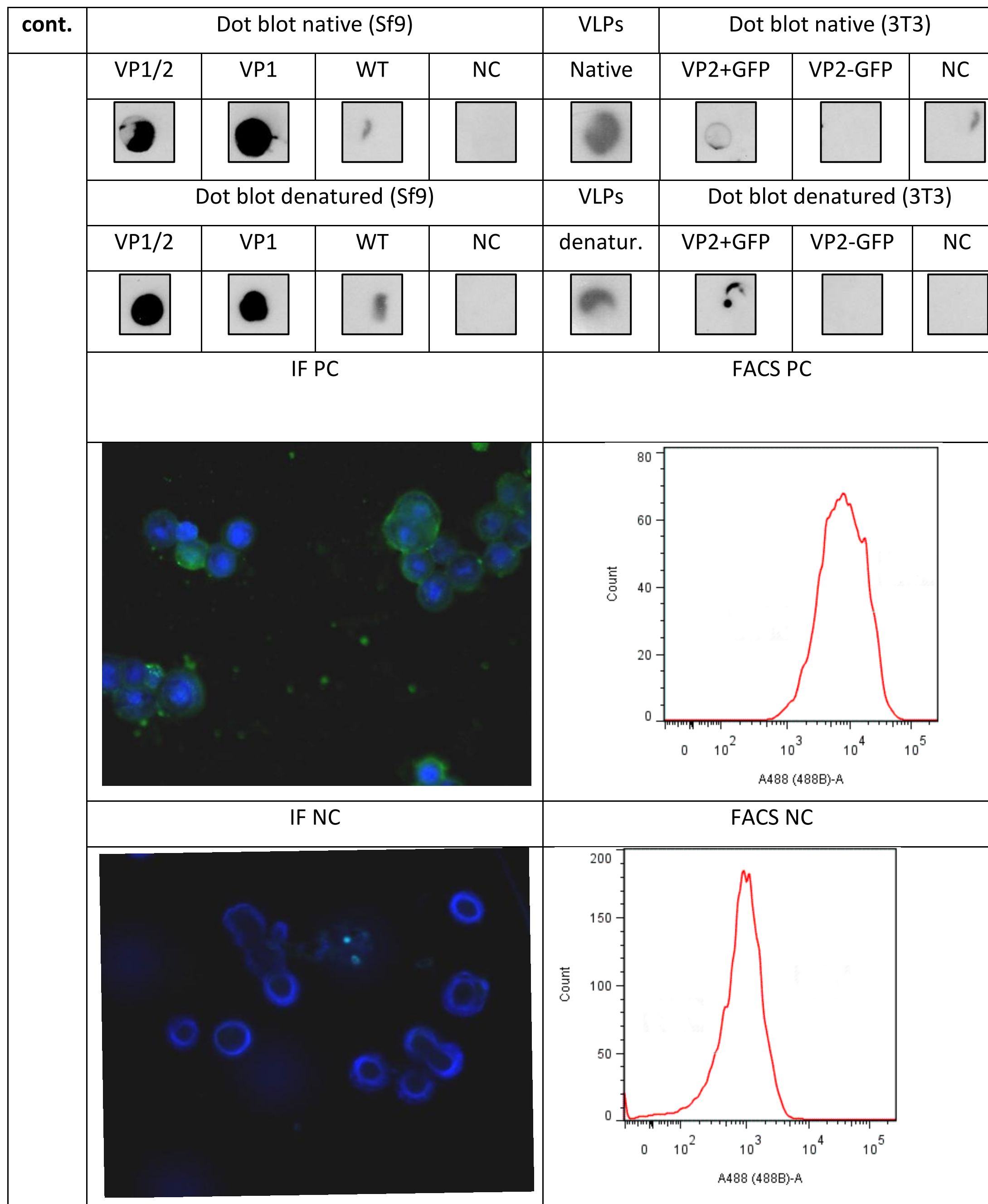


Fig. 4.10A: Testing of collected antibody from nine clones chosen for cultivation in larger scale. Various antigens were used as follows: **Dot blot native** or **denatured (Sf9)**: Sf9 cells infected with a recombinant baculovirus expressing VP1 and VP2 proteins of MCPyV (**VP1/2**) or VP1 protein of MCPyV (**VP1**) or with wild type baculovirus (**WT**) or mock infected (**NC**) were lysed in RIPA buffer (**native**) or boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). Purified VLPs consisting of VP1 and VP2 proteins of MCPyV were used (**VLPs Native**) or boiled for 10 minutes in concentrated Laemmli buffer (**VLPs denatur.**). Antigens were stained with monoclonal antibody against VP1 protein (structural epitope) of MCPyV (native samples) or polyclonal serum against VP1 protein of MCPyV (denatured samples). **Dot blot native** or **denatured (3T3)**: 3T3 cells were transfected with a plasmid enconding VP2 protein of MCPyV and GFP (**VP2+GFP**), VP2 protein of MCPyV (**VP2-GFP**) or mock transfected (**NC**), cells were lysed in RIPA buffer (**native**) or boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). Antigens were stained with antibody against GFP.

Immunofluorescent staining of fixed Sf9 cells infected with recombinant baculovirus producing VP1 and VP2 proteins of MCPyV (**IF PC**) or with wild type baculovirus (**IF NC**) was stained with monoclonal antibody against VP1 protein of MCPyV. FACS analysis: monoclonal antibody against VP1 protein of MCPyV (**FACS PC**) or blocking solution (**FACS NC**) were applied on intact Sf9 cells infected with recombinant baculovirus producing VP1 and VP2 proteins of MCPyV. Intensity of fluorescence (X axis) is shown in RFU (relative fluorescence units), count (Y axis) represents number of cells.

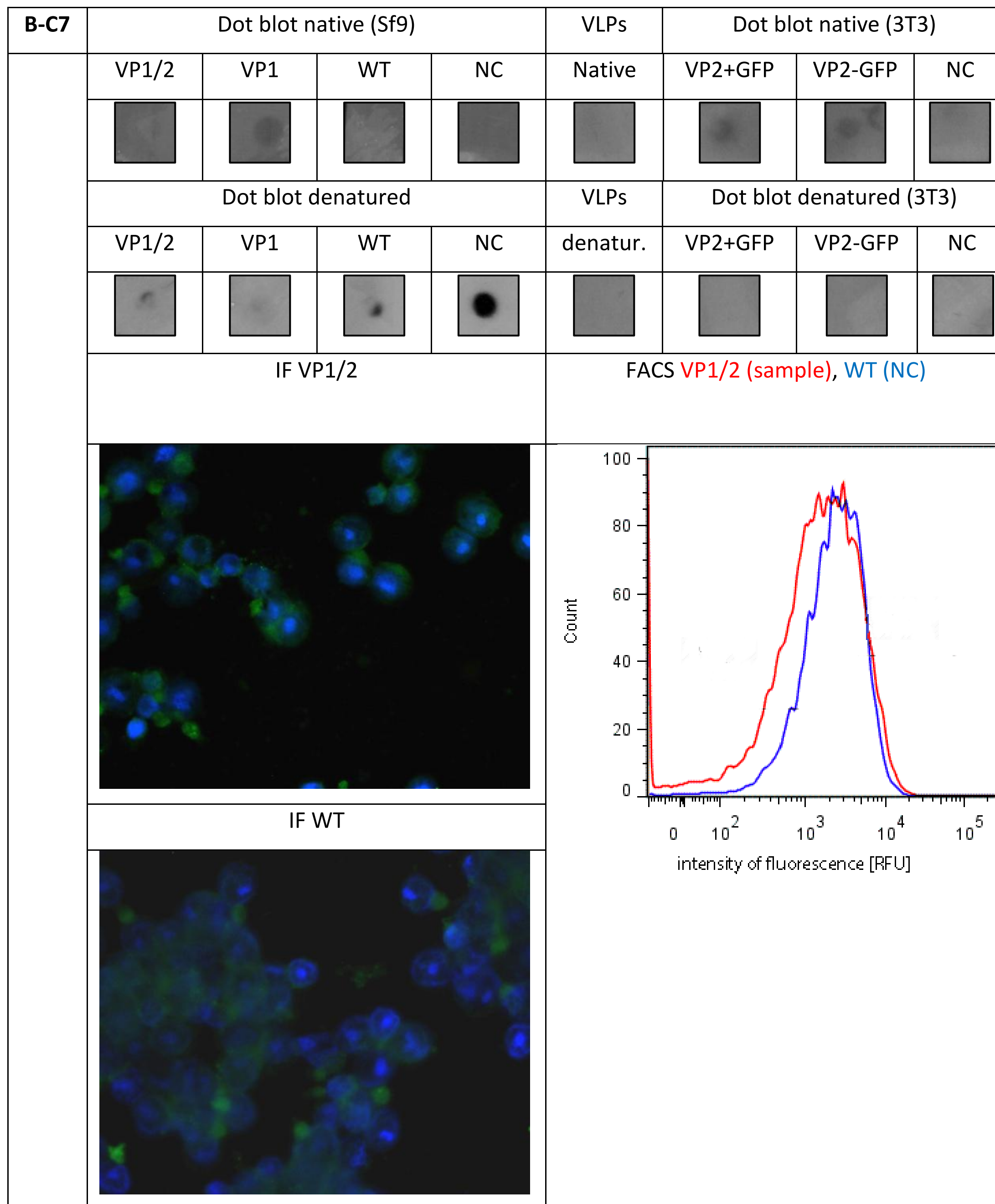


Fig. 4.10B: Testing of collected antibody from clone B-C7 (chosen for cultivation in larger scale). Culture medium was collected and used as primary antibody (staining overnight). Various antigens were used as follows: **Dot blot native** or **denatured (Sf9)**: Sf9 cells infected with a rombinant baculovirus expressing VP1 and VP2 proteins of MCPyV (**VP1/2**) or VP1 protein of MCPyV (**VP1**) or with wild type baculovirus (**WT**) or mock-infected (**NC**) were lysed in RIPA buffer (**native**) and boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). Purified VLPs consisting of VP1 and VP2 proteins of MCPyV were used (**VLPs Native**) or boiled for 10 minutes in concentrated Laemmli buffer (**VLPs denatur.**). **Dot blot native** or **denatured (3T3)**: 3T3 cells were transfected with a plasmid enconding VP2 protein of MCPyV and GFP (**VP2+GFP**), VP2 protein of MCPyV (**VP2-GFP**) or mock transfected (**NC**), cells were lysed in RIPA buffer (**native**) or boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). Immunofluorescent staining was performed on fixed Sf9 cells infected with recombinant baculovirus producing VP1 and VP2 proteins of MCPyV (**IF VP1/2**) or with wild type baculovirus (**IF**

WT). FACS analysis was used to test staining of intact Sf9 cells infected with recombinant baculovirus producing VP1 and VP2 proteins of MCPyV (**VP1/2(sample)**, red curve), or infected with wild type baculovirus (**WT (NC)**, blue curve). Intensity of fluorescence (X axis) is shown in RFU (relative fluorescence units), count (Y axis) represents number of cells.

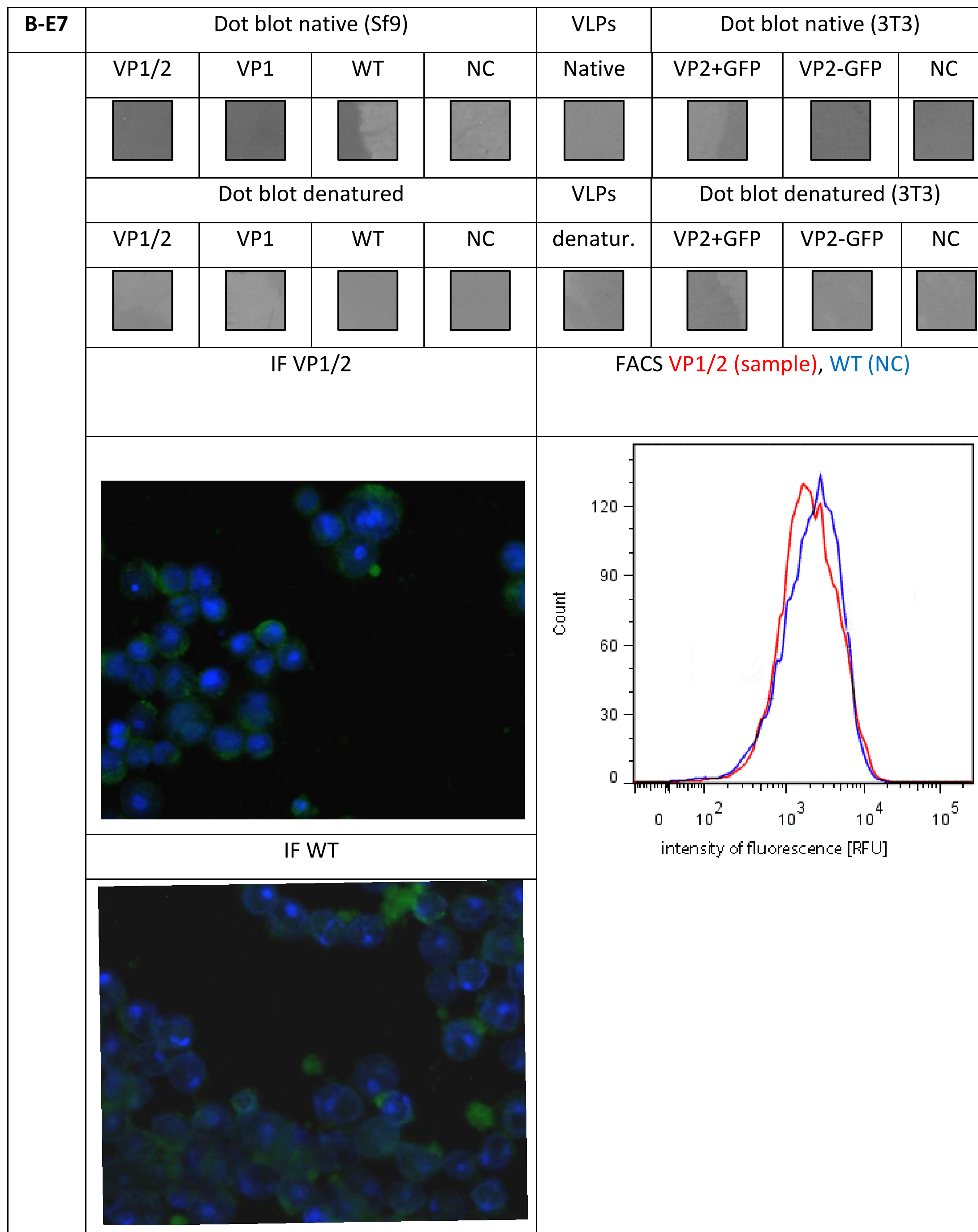


Fig. 4.10C: Testing of collected antibody from clone B-E7 (chosen for cultivation in larger scale). Culture medium was collected and used as primary antibody (staining overnight). Various antigens were used as follows: **Dot blot native** or **denatured (Sf9)**: Sf9 cells infected with a rombinant baculovirus expressing VP1 and VP2 proteins of MCPyV (**VP1/2**) or VP1 protein of MCPyV (**VP1**) or with wild type baculovirus (**WT**) or mock-infected (**NC**) were lysed in RIPA buffer (**native**) and boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). Purified VLPs consisting of VP1 and VP2 proteins of MCPyV were used (**VLPs Native**) or boiled for 10 minutes in concentrated Laemmli buffer (**VLPs denatur.**). **Dot blot native** or **denatured (3T3)**: 3T3 cells were transfected with a plasmid enconding VP2 protein of MCPyV and GFP (**VP2+GFP**), VP2 protein of MCPyV (**VP2-GFP**) or mock transfected (**NC**), cells were lysed in RIPA buffer (**native**) or boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). Immunofluorescent staining was performed on fixed Sf9 cells infected with

recombinant baculovirus producing VP1 and VP2 proteins of MCPyV (**IF VP1/2**) or with wild type baculovirus (**IF WT**). FACS analysis was used to test staining of intact Sf9 cells infected with recombinant baculovirus producing VP1 and VP2 proteins of MCPyV (**VP1/2(sample)**, red curve), or infected with wild type baculovirus (**WT (NC)**, blue curve). Intensity of fluorescence (X axis) is shown in RFU (relative fluorescence units), count (Y axis) represents number of cells.

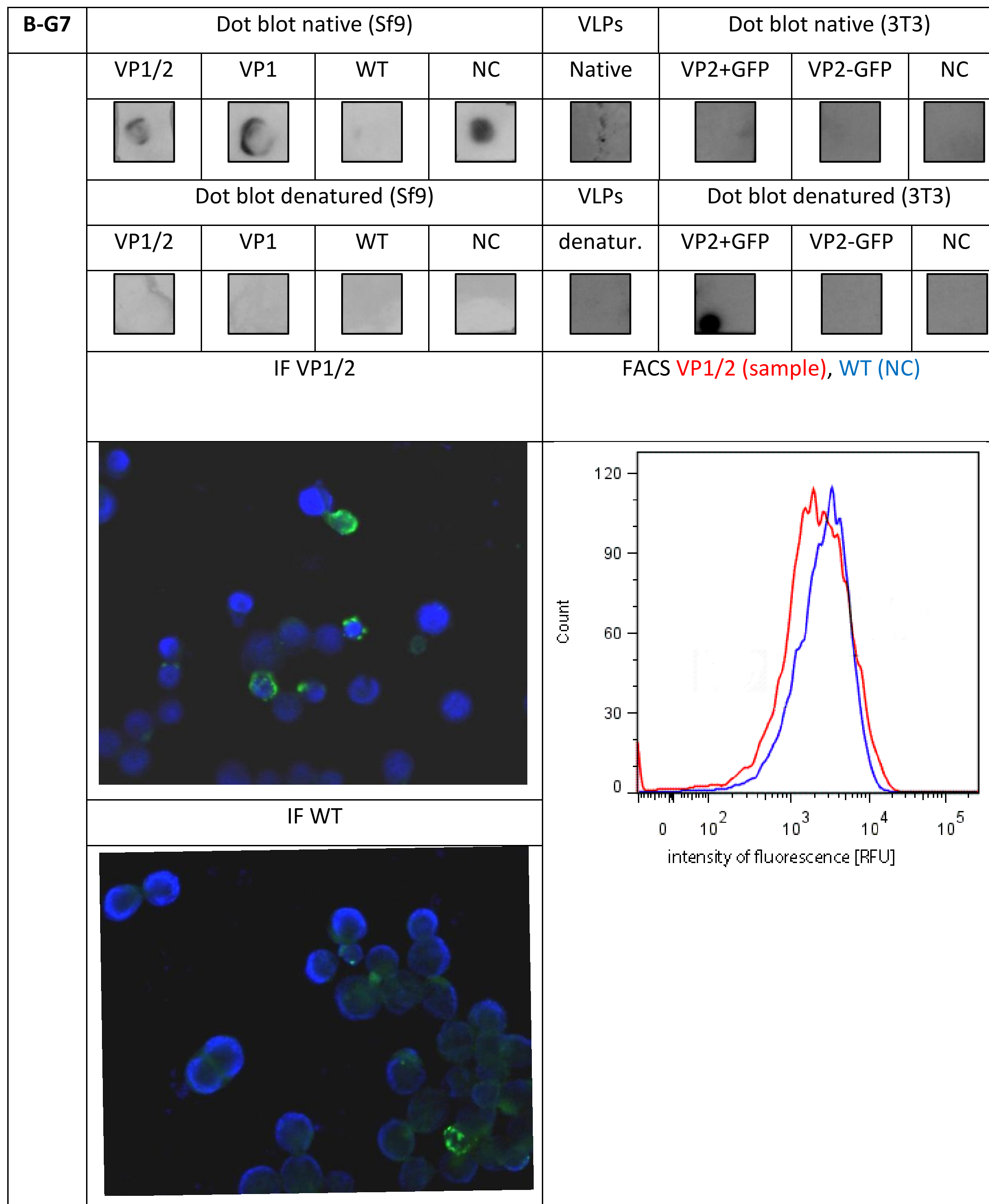


Fig. 4.10D: Testing of collected antibody from clone B-G7 (chosen for cultivation in larger scale). Culture medium was collected and used as primary antibody (staining overnight). Various antigens were used as follows: **Dot blot native** or **denatured (Sf9)**: Sf9 cells infected with a rombinant baculovirus expressing VP1 and VP2 proteins of MCPyV (**VP1/2**) or VP1 protein of MCPyV (**VP1**) or with wild type baculovirus (**WT**) or mock-infected (**NC**) were lysed in RIPA buffer (**native**) and boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). Purified VLPs consisting of VP1 and VP2 proteins of MCPyV were used (**VLPs Native**) or boiled for 10 minutes in concentrated Laemmli buffer (**VLPs denatur.**). **Dot blot native** or **denatured (3T3)**: 3T3 cells were transfected with a plasmid enconding VP2 protein of MCPyV and GFP (**VP2+GFP**), VP2 protein of MCPyV (**VP2-GFP**) or mock transfected (**NC**), cells were lysed in RIPA buffer (**native**) or boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). Immunofluorescent staining was performed on fixed Sf9 cells infected with recombinant baculovirus producing VP1 and VP2 proteins of MCPyV (**IF VP1/2**) or with wild type baculovirus (**IF**

WT). FACS analysis was used to test staining of intact Sf9 cells infected with recombinant baculovirus producing VP1 and VP2 proteins of MCPyV (**VP1/2(sample)**, red curve), or infected with wild type baculovirus (**WT (NC)**, blue curve). Intensity of fluorescence (X axis) is shown in RFU (relative fluorescence units), count (Y axis) represents number of cells.

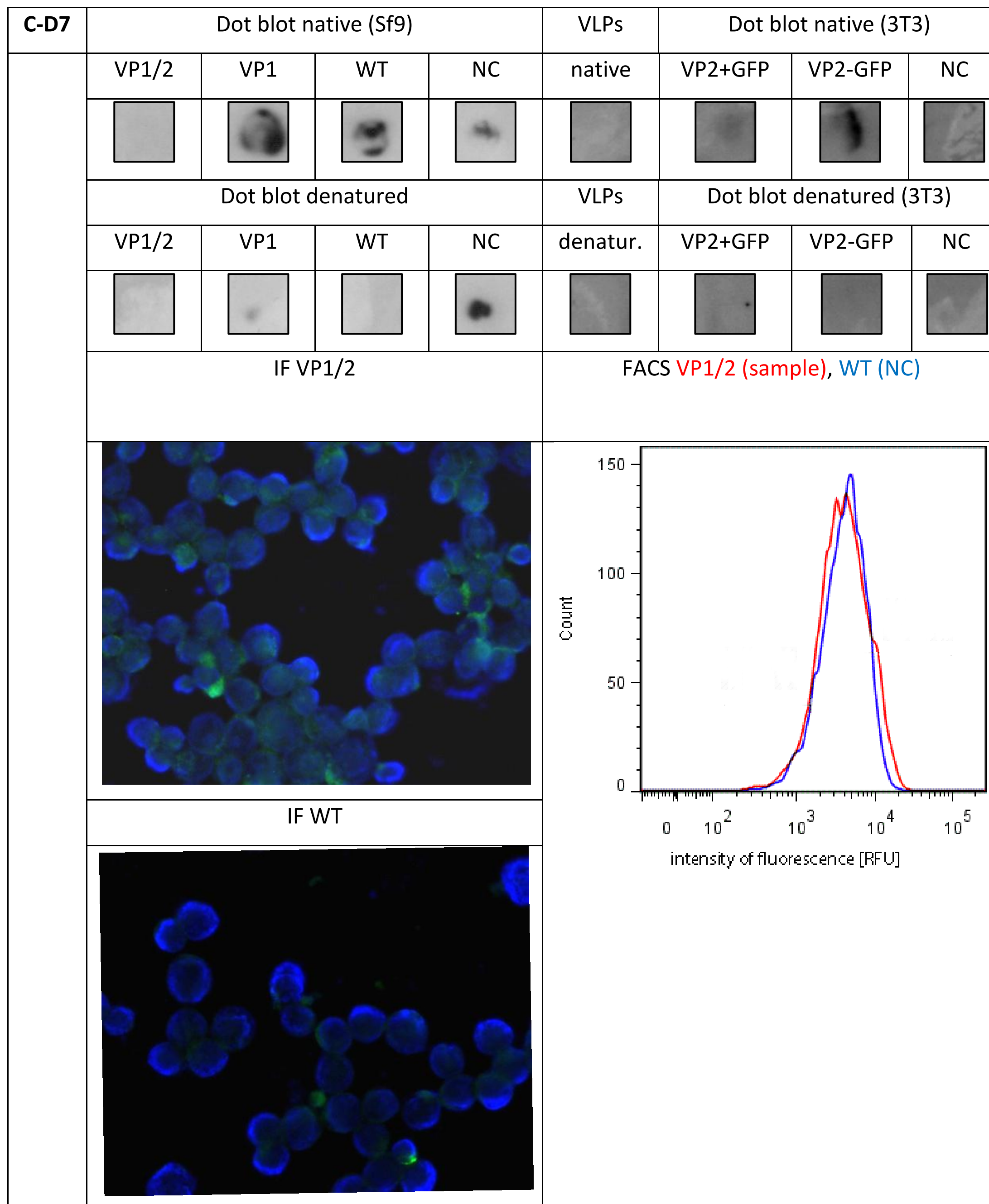


Fig. 4.10E: Testing of collected antibody from clone C-D7 (chosen for cultivation in larger scale). Culture medium was collected and used as primary antibody (staining overnight). Various antigens were used as follows: **Dot blot native** or **denatured (Sf9)**: Sf9 cells infected with a rombinant baculovirus expressing VP1 and VP2 proteins of MCPyV (**VP1/2**) or VP1 protein of MCPyV (**VP1**) or with wild type baculovirus (**WT**) or mock-infected (**NC**) were lysed in RIPA buffer (**native**) and boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). Purified VLPs consisting of VP1 and VP2 proteins of MCPyV were used (**VLPs Native**) or boiled for 10 minutes in concentrated Laemmli buffer (**VLPs denatur.**). **Dot blot native** or **denatured (3T3)**: 3T3 cells were transfected with a plasmid enconding VP2 protein of MCPyV and GFP (**VP2+GFP**), VP2 protein of MCPyV (**VP2-GFP**) or mock transfected (**NC**), cells were lysed in RIPA buffer (**native**) or boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). Immunofluorescent staining was performed on fixed Sf9 cells infected with recombinant baculovirus producing VP1 and VP2 proteins of MCPyV (**IF VP1/2**) or with wild type baculovirus (**IF**

WT). FACS analysis was used to test staining of intact Sf9 cells infected with recombinant baculovirus producing VP1 and VP2 proteins of MCPyV (**VP1/2(sample)**, red curve), or infected with wild type baculovirus (**WT (NC)**, blue curve). Intensity of fluorescence (X axis) is shown in RFU (relative fluorescence units), count (Y axis) represents number of cells.

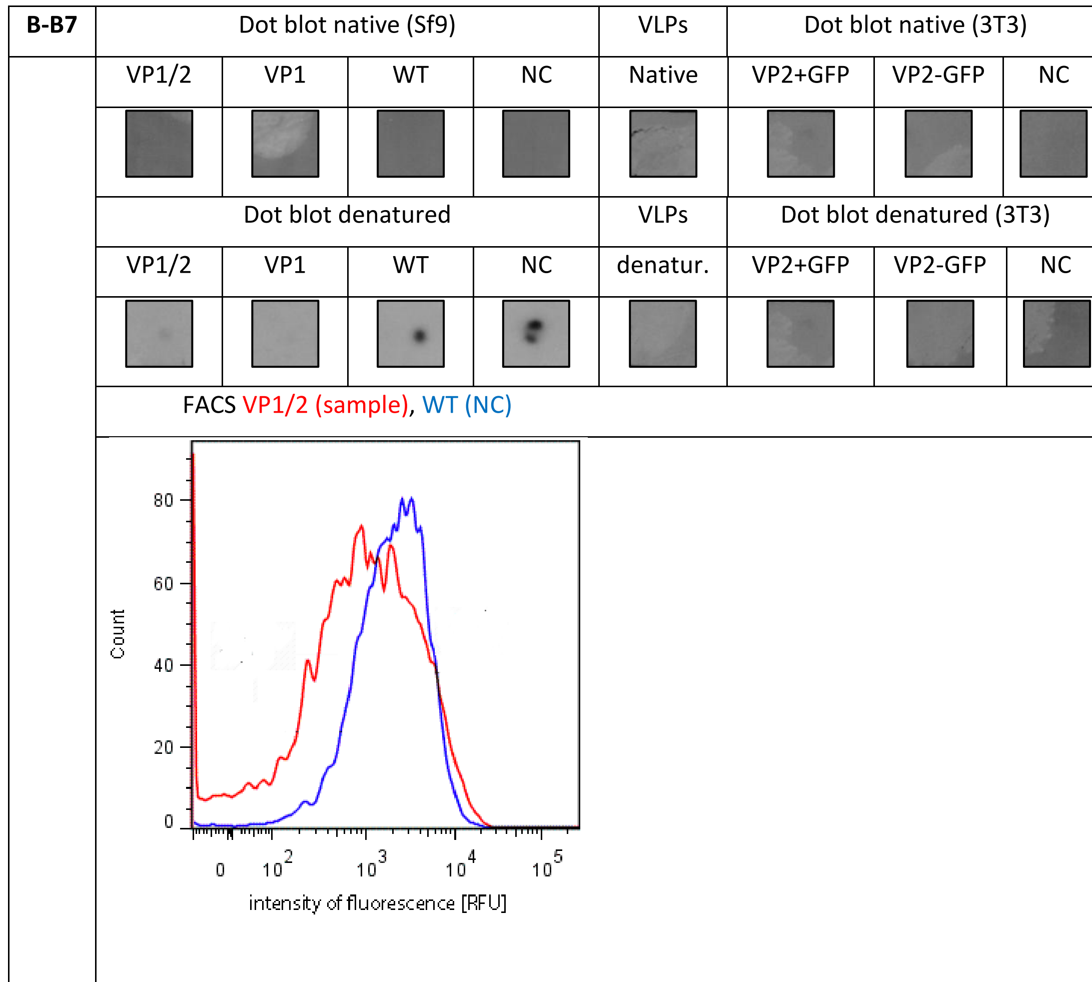


Fig. 4.10F: Testing of collected antibody from clone B-B7 (chosen for cultivation in larger scale). Culture medium was collected and used as primary antibody (staining overnight). Various antigens were used as follows: **Dot blot native** or **denatured (Sf9)**: Sf9 cells infected with a rombinant baculovirus expressing VP1 and VP2 proteins of MCPyV (**VP1/2**) or VP1 protein of MCPyV (**VP1**) or with wild type baculovirus (**WT**) or mock-infected (**NC**) were lysed in RIPA buffer (**native**) and boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). Purified VLPs consisting of VP1 and VP2 proteins of MCPyV were used (**VLPs Native**) or boiled for 10 minutes in concentrated Laemmli buffer (**VLPs denatur.**). **Dot blot native** or **denatured (3T3)**: 3T3 cells were transfected with a plasmid enconding VP2 protein of MCPyV and GFP (**VP2+GFP**), VP2 protein of MCPyV (**VP2-GFP**) or mock transfected (**NC**), cells were lysed in RIPA buffer (**native**) or boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). FACS analysis was used to test staining of intact Sf9 cells infected with recombinant baculovirus producing VP1 and VP2 proteins of MCPyV (**VP1/2(sample)**, red curve), or infected with wild type baculovirus (**WT (NC)**, blue curve). Intensity of fluorescence (X axis) is shown in RFU (relative fluorescence units), count (Y axis) represents number of cells.

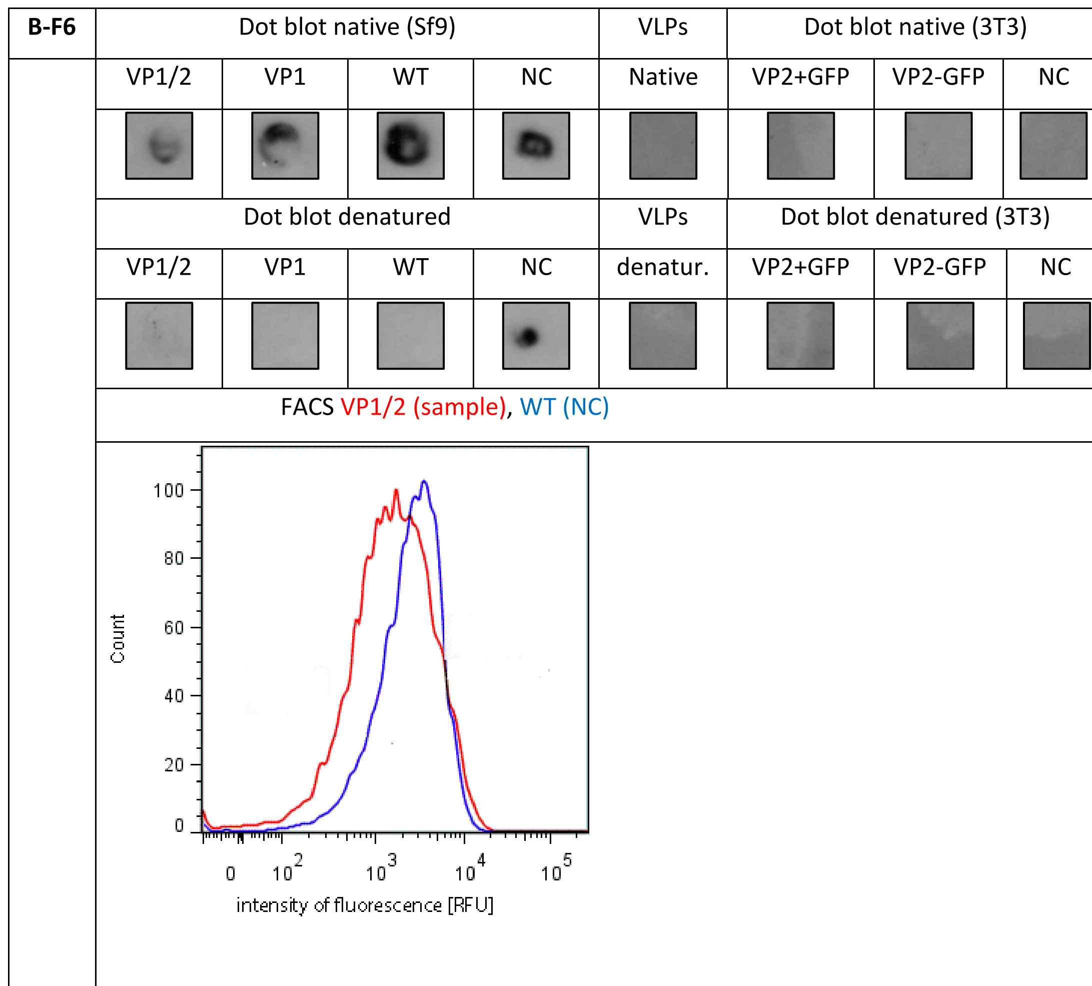


Fig. 4.10G: Testing of collected antibody from clone B-F6 (chosen for cultivation in larger scale). Culture medium was collected and used as primary antibody (staining overnight). Various antigens were used as follows: **Dot blot native** or **denatured (Sf9)**: Sf9 cells infected with a rombinant baculovirus expressing VP1 and VP2 proteins of MCPyV (**VP1/2**) or VP1 protein of MCPyV (**VP1**) or with wild type baculovirus (**WT**) or mock-infected (**NC**) were lysed in RIPA buffer (**native**) and boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). Purified VLPs consisting of VP1 and VP2 proteins of MCPyV were used (**VLPs Native**) or boiled for 10 minutes in concentrated Laemmli buffer (**VLPs denatur.**). **Dot blot native** or **denatured (3T3)**: 3T3 cells were transfected with a plasmid enconding VP2 protein of MCPyV and GFP (**VP2+GFP**), VP2 protein of MCPyV (**VP2-GFP**) or mock transfected (**NC**), cells were lysed in RIPA buffer (**native**) or boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). FACS analysis was used to test staining of intact Sf9 cells infected with recombinant baculovirus producing VP1 and VP2 proteins of MCPyV (**VP1/2(sample)**, red curve), or infected with wild type baculovirus (**WT (NC)**, blue curve). Intensity of fluorescence (X axis) is shown in RFU (relative fluorescence units), count (Y axis) represents number of cells.

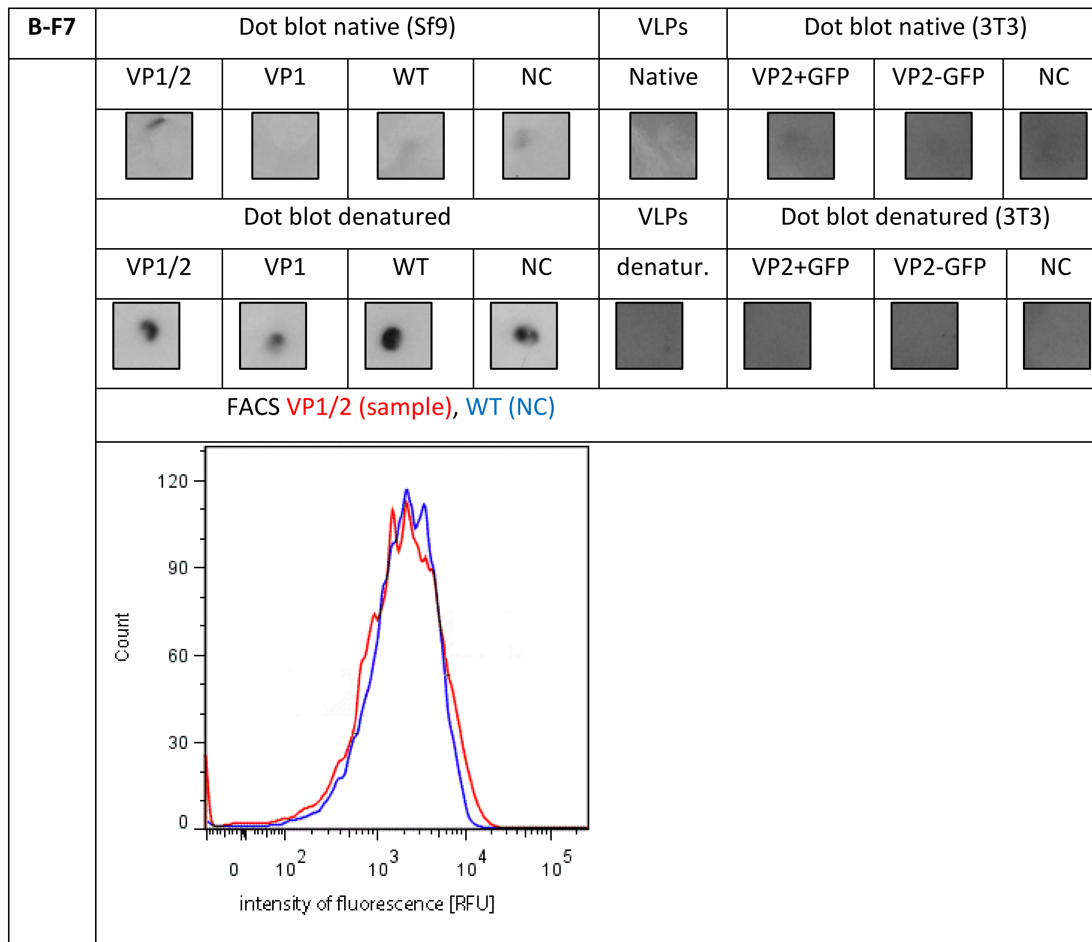


Fig. 4.10H: Testing of collected antibody from clone B-F7 (chosen for cultivation in larger scale). Culture medium was collected and used as primary antibody (staining overnight). Various antigens were used as follows: **Dot blot native** or **denatured (Sf9)**: Sf9 cells infected with a rombinant baculovirus expressing VP1 and VP2 proteins of MCPyV (**VP1/2**) or VP1 protein of MCPyV (**VP1**) or with wild type baculovirus (**WT**) or mock-infected (**NC**) were lysed in RIPA buffer (**native**) and boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). Purified VLPs consisting of VP1 and VP2 proteins of MCPyV were used (**VLPs Native**) or boiled for 10 minutes in concentrated Laemmli buffer (**VLPs denatur.**). **Dot blot native** or **denatured (3T3)**: 3T3 cells were transfected with a plasmid enconding VP2 protein of MCPyV and GFP (**VP2+GFP**), VP2 protein of MCPyV (**VP2-GFP**) or mock transfected (**NC**), cells were lysed in RIPA buffer (**native**) or boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). FACS analysis was used to test staining of intact Sf9 cells infected with recombinant baculovirus producing VP1 and VP2 proteins of MCPyV (**VP1/2(sample)**, red curve), or infected with wild type baculovirus (**WT (NC)**, blue curve). Intensity of fluorescence (X axis) is shown in RFU (relative fluorescence units), count (Y axis) represents number of cells.

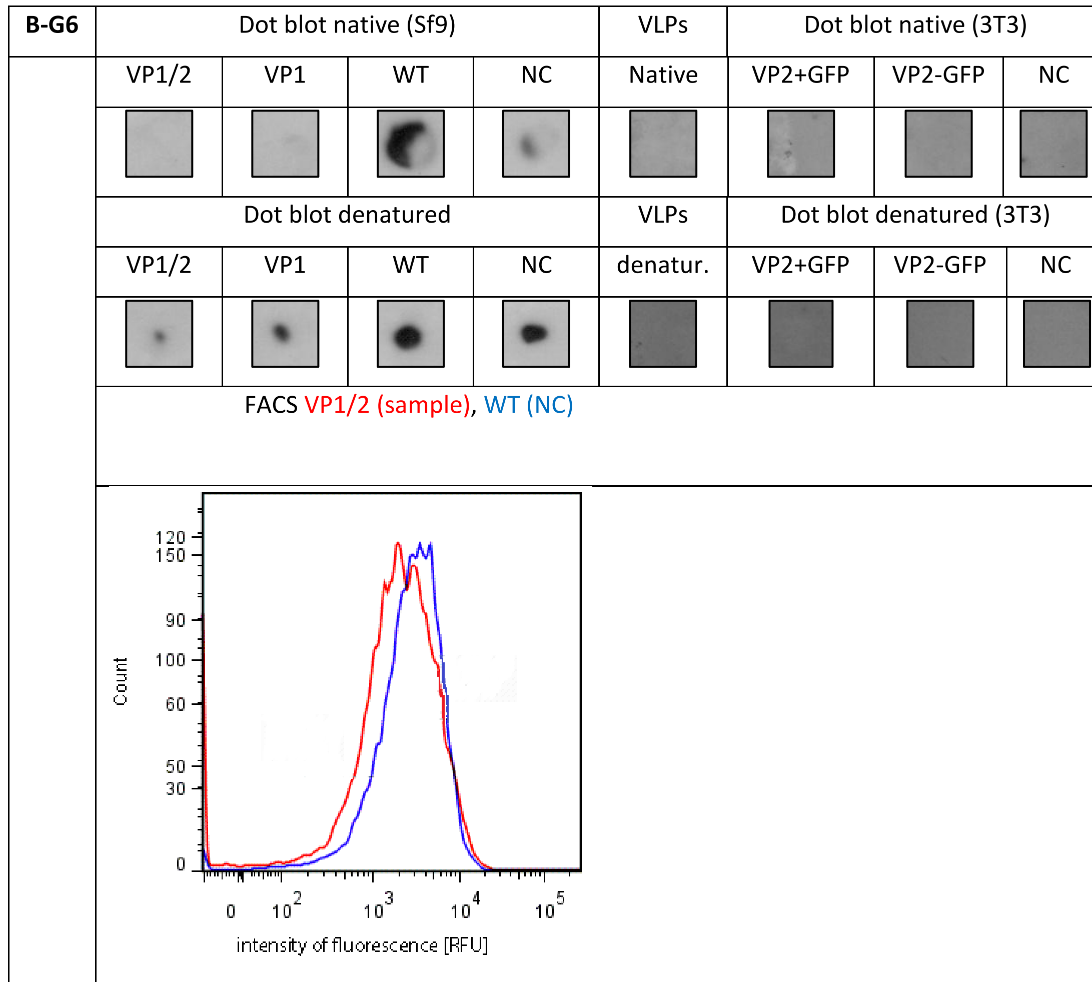


Fig. 4.10l: Testing of collected antibody from clone B-G6 (chosen for cultivation in larger scale). Culture medium was collected and used as primary antibody (staining overnight). Various antigens were used as follows: **Dot blot native** or **denatured (Sf9)**: Sf9 cells infected with a rombinant baculovirus expressing VP1 and VP2 proteins of MCPyV (**VP1/2**) or VP1 protein of MCPyV (**VP1**) or with wild type baculovirus (**WT**) or mock-infected (**NC**) were lysed in RIPA buffer (**native**) and boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). Purified VLPs consisting of VP1 and VP2 proteins of MCPyV were used (**VLPs Native**) or boiled for 10 minutes in concentrated Laemmli buffer (**VLPs denatur.**). **Dot blot native** or **denatured (3T3)**: 3T3 cells were transfected with a plasmid enconding VP2 protein of MCPyV and GFP (**VP2+GFP**), VP2 protein of MCPyV (**VP2-GFP**) or mock transfected (**NC**), cells were lysed in RIPA buffer (**native**) or boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). FACS analysis was used to test staining of intact Sf9 cells infected with recombinant baculovirus producing VP1 and VP2 proteins of MCPyV (**VP1/2(sample)**, red curve), or infected with wild type baculovirus (**WT (NC)**, blue curve). Intensity of fluorescence (X axis) is shown in RFU (relative fluorescence units), count (Y axis) represents number of cells.

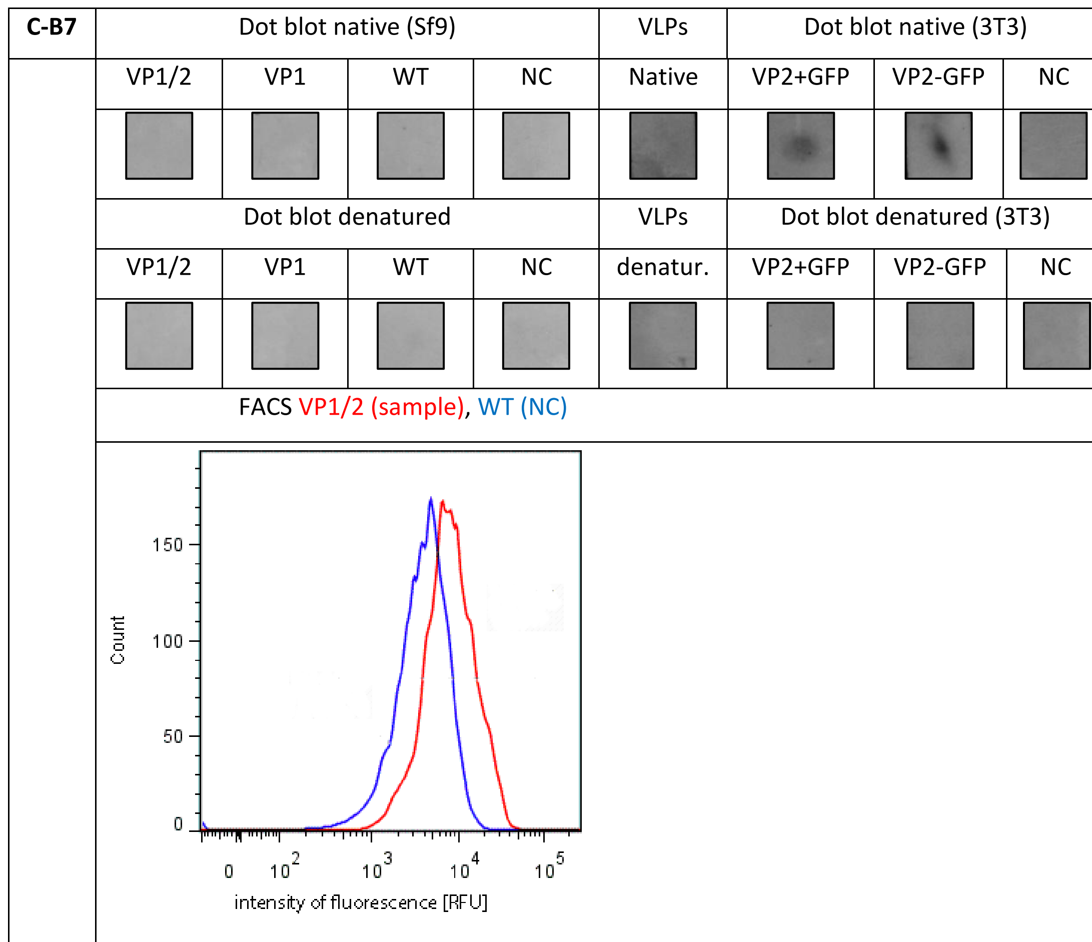


Fig. 4.10J: Testing of collected antibody from clone C-B7 (chosen for cultivation in larger scale). Culture medium was collected and used as primary antibody (staining overnight). Various antigens were used as follows: **Dot blot native** or **denatured (Sf9)**: Sf9 cells infected with a rombinant baculovirus expressing VP1 and VP2 proteins of MCPyV (**VP1/2**) or VP1 protein of MCPyV (**VP1**) or with wild type baculovirus (**WT**) or mock-infected (**NC**) were lysed in RIPA buffer (**native**) and boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). Purified VLPs consisting of VP1 and VP2 proteins of MCPyV were used (**VLPs Native**) or boiled for 10 minutes in concentrated Laemmli buffer (**VLPs denatur.**). **Dot blot native** or **denatured (3T3)**: 3T3 cells were transfected with a plasmid enconding VP2 protein of MCPyV and GFP (**VP2+GFP**), VP2 protein of MCPyV (**VP2-GFP**) or mock transfected (**NC**), cells were lysed in RIPA buffer (**native**) or boiled for 10 minutes in concentrated Laemmli buffer (**denatured**). FACS analysis was used to test staining of intact Sf9 cells infected with recombinant baculovirus producing VP1 and VP2 proteins of MCPyV (**VP1/2(sample)**, red curve), or infected with wild type baculovirus (**WT (NC)**, blue curve). Intensity of fluorescence (X axis) is shown in RFU (relative fluorescence units), count (Y axis) represents number of cells.

4.3. Preparation of antigen, truncated VP2 protein from BKV, for antibody preparation

Even though our laboratory conducts research on BKV life cycle, we still do not possess specific monoclonal antibody against BKV minor proteins. Such antibody is neither available commercially. Cross-reactive polyclonal antibodies against minor proteins of other polyomaviruses are used instead, but their specificity is insufficient. Due to insuperable difficulties with expression of VP2 protein in an expression system, experienced in our laboratory earlier, truncation of the protein was proposed. The protein was truncated on C-terminus, avoiding the most hydrophobic domain of VP2 (Fig. 4.11).

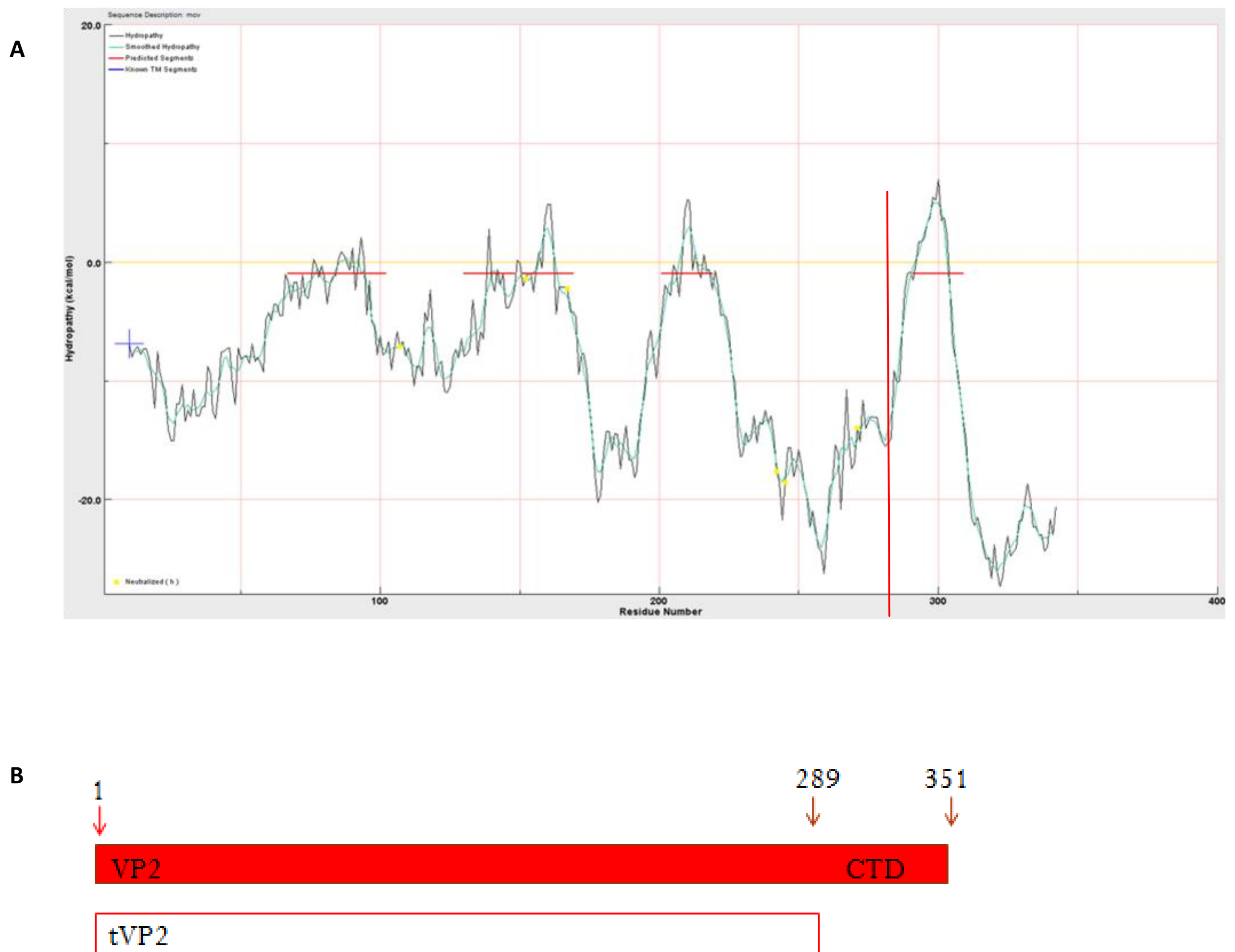


Fig.4.11: A: Transmembrane domain prediction calculated for protein VP2 of BKV in Membrane Protein Explorer. X axis represent amino acid residues (1 - 351), Y axis represents protein hydrophobicity, horizontal red line segments represent predicted transmembrane domains, red vertical line represents site of truncation (eliminating C-terminus with the most hydrophobic part). **B: Scheme of protein VP2 truncation** (eliminating the most hydrophobic C-terminal domain, CTD).

4.3.1. Expression *in silico*

Plasmid pET29b was chosen for expression of tVP2 (Fig. 4.12). Expression *in silico* was performed to verify conservation of correct open reading frame (ORF) in the plasmid design. Insert sequence was put in the sequence of plasmid pET29b. Translation *in silico* was performed in ExPASy Translate Tool (Fig. 4.13). Translation start, sequence of tVP2, S-tag and His-tag shared the same ORF.

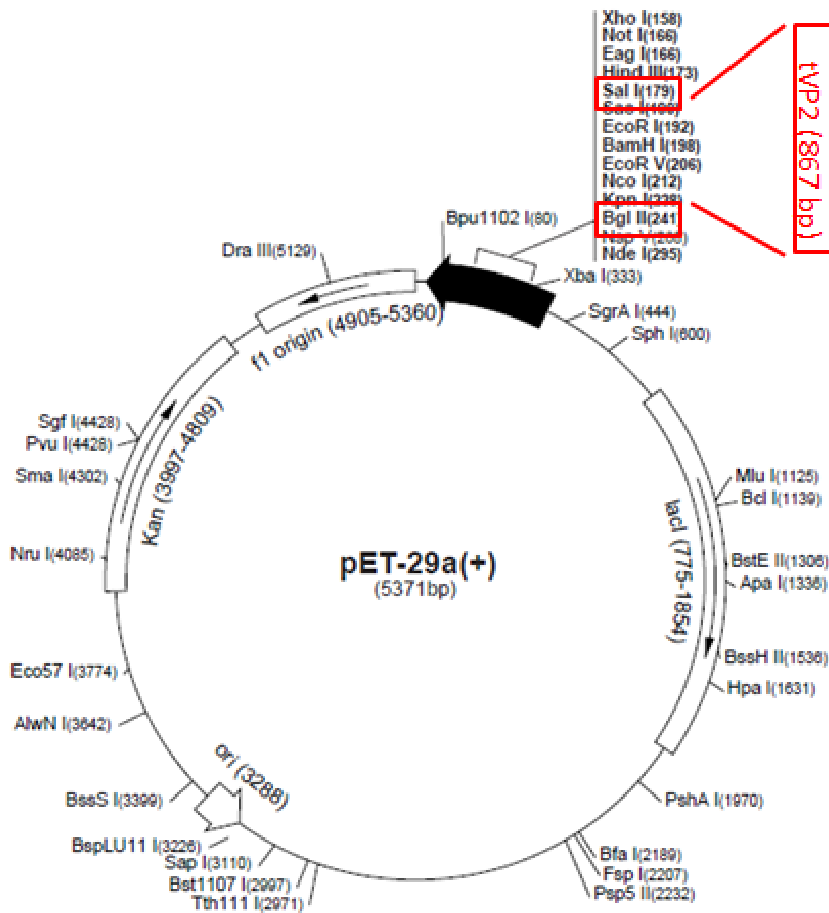


Fig.4.12: Plasmid pET29 with in tVP2 insert, restriction sites of SalI and BglII restriction endonucleases were used for cloning.

A:

ATGAAAGAAACCGCTGCTGCTAAATTCGAACGCCAGCACATGGACAGCCCAGATCTTATGGGTGCTGCTCTAGCACTTTTGGGG
GACCTAGTTGCCAGTGTATCTGAGGCTGCTGCTGCCACAGGATTTTCAGTGGCTGAAATTGCTGCTGGGGAGGCTGCTGCTGCT
ATAGAAGTTCAAATTGCATCCCTTGCTACTGTAGAGGGCATAACAAGTACCTCAGAGGCTATAGCTGCTATAGGCCTAACTCCTC
AAACATATGCTGTAATTGCTGGTGCTCCTGGGGCTATTGCTGGGTTTGCTGCTTTAATTCAAACTGTTAGTGGTATTAGTTCCTTG
GCTCAAGTAGGGTATAGGTTCTTTAGTGATTGGGATCACAAAGTTTCCACTGTAGGCCTCTATCAGCAATCAGGCATGGCTTTGG
AATTGTTTAACCCAGATGAGTACTATGATATTCTGTTTCCTGGTGTAAATACTTTTGTTAATAATATTCAATACCTTGATCCTAGGC
ATTGGGGTCCTTCTTTGTTTGCTACTATTTCCCAGGCTTTGTGGCATGTTATTAGGGATGATATACCTTCTATAACCTCACAGGAAT
TGCAGAGAAGAACAGAAAGATTTTTTAGAGACTCCTTGGCTAGATTTTTGGAGGAAACTACCTGGACAATTGTAAATGCCCCTAT
AAACTTTTATAATTATATTCAACAATATTATTCTGATCTTTCCCCTATTAGGCCCTCAATGGTTAGACAAGTAGCTGAAAGGGAAG
GTACCCGTGTACATTTTGGCCATACTTATAGTATAGATGATGCTGACAGTATAGAAGAAGTTACACAAAGAATGGACTTAAGAAA
TCAACAAAGTGTACATTCAGGAGAGTTTATAGAAAAAACTATTGCCCCAGGAGGTGCTAATCAAAGAACTGTCGACAAGCTTGC
GGCCGCACTCGAGCACCACCACCACCACCACTGA

B:

MKETAAAKFERQHMDSPDLMGAALALLGDLVASVSEAAAATGFSVAEIAAGEAAAAIEVQIASLATVEGITSTSEAIAAIGLTPQTYAVI
AGAPGAIAGFAALIQTVSGISSLAQVGYRFFSDWDHKVSTVGLYQQSGMALELFNPDEYYDILFPGVNTFVNNIQYLDPRHWGPSLFA
TISQALWHVIRDDIPSITSQELQRRTERFFRDSLARFLEETTWTIVNAPINFYNYIQQYYSDLSPIRPSMVRQVAEREGTRVHFGHTYSID
DADSIEEVTQRMDLRNQQSVHSGEFIEKTIAPGGANQRTVDKLAAALEHHHHHH-

Fig.4.13: Expression *in silico*, nucleotide sequence of tVP2 DNA (blue) was put in the sequence of pET29b multiple cloning site (shown from intiation to stop codons (underlined), sequence of His-tag (red) and S-tag (green)). The whole sequence was translated in ExPASy Translate Tool. Resultant amino acid sequence is shown in (B). Both sequences contained tVP2 (blue), S-tag (green) and His-tag (red) in the same ORF and no undesirable stop codons. Residual sequences of pET29b multiple cloning site are shown in black.

4.3.2. Preparation of recombinant plasmid

Plasmid pET29b was isolated using QIAprep Spin Miniprep Kit (Qiagen) for this purpose. DNA concentration (measured by Nanodrop) was 64.7 ng/µl. Plasmid was cleaved by restriction endonucleases SalI and BglII (Fig. 4.14) and dephosphorylated to avoid further vector religation. DNA coding for desired part of VP2 protein (tVP2) was prepared by PCR and restriction cleaving (see chapter 3.1.7. for primers and 3.2.3.6. for details of the procedure) and cloned into plasmid pET29b.

Ligation mix was prepared in molar ratio 1:3 (plasmid : insert). Ligation mix and a negative control (ligation mix with no added insert) were electroporated into XL1 blue bacteria. Negative control contained about twice lower amount of colonies in comparison with the amount of colonies on plates with sample bacteria.

Twelve colonies were tested for presence of tVP2 insert. Plasmid DNA was isolated by minipreparation from bacteria cultivated overnight and insertion was verified by cleavage with SalI and BglII restriction endonucleases. Out of twelve colonies tested, two (colonies 11 and 12) were identified to contain plasmid with insert (Fig. 4.15). Insertion part (tVP2 DNA) in plasmids isolated from the selected colonies were further sequenced (see supplement material, Fig. S1). The insert sequence was proven to be identical to template sequence. The whole plasmid sequence was not sequenced due to its excessive length. Expression *in silico* was performed again with the sequences obtained from sequencing, with the same result as in chapter 4.3.1.

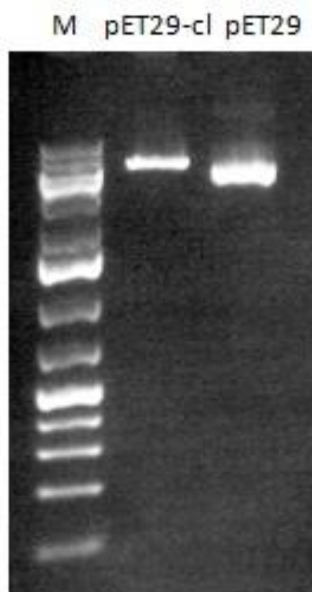


Fig.4.14: Plasmid pET29b after cleavage with restriction endonucleases BglII and SalI (pET29-cl) is less mobile and does not occur in two isoformes as non-cleaved plasmid (pET29).M: marker.

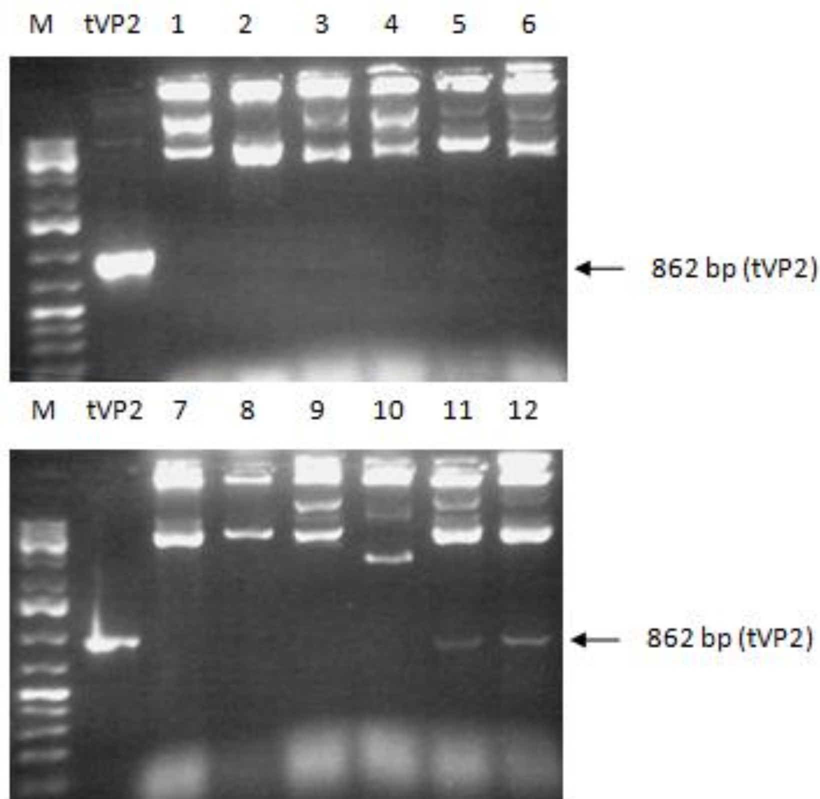


Fig.4.15: Restriction analysis of prepared recombinant plasmid pET29b-tVP2. Agarose electrophoresis for verification of plasmids isolated from monocolonies after electroporation of ligation mix with tVP2 DNA in pET29b. M: marker, tVP2 PCR product used as insert was used as positive control (tVP2). Lines 1-12: Plasmids pET29b, isolated from bacterial monocolonies, after endonuclease digestion. DNA was isolated by minipreparation (see chapter 3.2.3.2.1. for details of the procedure) and cleaved using BglII and SalI. Cleavage of plasmid with correct insert resulted in presence of two fragments – high molecular (pET29b plasmid) and 862 bp long (tVP2 insert). Correct insertion can be seen in colonies 11 and 12 (lines 11, 12).

4.3.3. Expression of protein

Bacteria BL-21(DE3) were used for protein expression. Plasmid pET29b with inserted tVP2 DNA was isolated using the Qiagen kit from bacteria XL1 blue (colony 12) cultivated overnight. Plasmid was electroporated into BL-21(DE3) bacteria and these were applied on agar plates with kanamycin and cultivated overnight.

Three monocolonies were inoculated into 2xYT medium with kanamycin (see chapter 3.1.11.1. for contents) and were incubated overnight. In the morning bacteria samples were diluted to OD_{600} = 0.1 and were cultivated until OD_{600} reached ~0.7. Then, T7 RNA polymerase expression was induced by IPTG addition (final concentration 0.1 mM). Since bacteria contained plasmid pET29b with inserted tVP2 DNA under T7 polymerase promoter, plasmid transcription is performed by T7 RNA polymerase. Bacteria were cultivated at 22°C for 2 or 3 hours.

To gather produced tVP2 protein, bacteria were harvested by centrifugation for 10 minutes at 4000 g in a refrigerated centrifuge. To specify in which protein fraction tVP2 was present, two types of protein samples were prepared by lysis. Native lysis (freezing in liquid nitrogen, see chapter 3.2.5.2. for details of the procedure) reveals only PBS-soluble proteins. Cell lysis (boiling in Laemmli buffer, see chapter 3.2.5.1. for details of the procedure) reveals all proteins (soluble and insoluble in PBS). Prepared lysates were tested by Western blot (Fig. 4.16). No signal was detected in samples using this method. Due to absence of signal, the whole set of samples was repeatadely tested by dot blot which is faster and more convenient for testing of bigger sample sets (Fig. 4.17). Using this method, weak VP2-specific signal was detected in cell lysates, implying only low concentrations of desired protein.

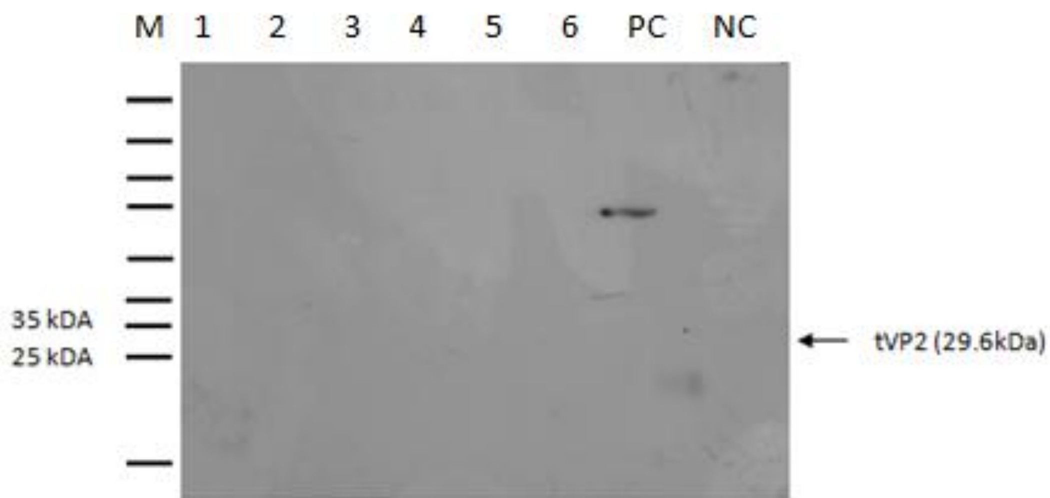


Fig.4.16: **Production of tVP2 protein in bacterial culture after IPTG induction.** Detection of His-tag on Western blot of bacterial lysates; lines 1 and 4: native lysates of BL21 after induced expression of tVP2, lines 2 and 5: cell lysates of BL21 after induced expression of tVP2, lines 3 and 6: control non-induced cell lysates of BL21. Samples were prepared in two parallel experiments (1,2,3 and 4,5,6). PC: A protein of MPyV VP1 fused with CAP and His-tag was used as positive control (kindly provided by M. Fraiberk). NC: Cell lysate of BL-21 bacteria with no added plasmid was used as negative control. M: marker. Primary antibody: polyclonal antibody against His-tag (dilution 1:100). Secondary antibody: goat against rabbit fused with HRP (dilution 1:1000). Film was exposed for 15 minutes. Protein tVP2 fused with S-tag and His-tag has molecular weight of 29.6 kDa, no signal is visible for experimental bacteria.

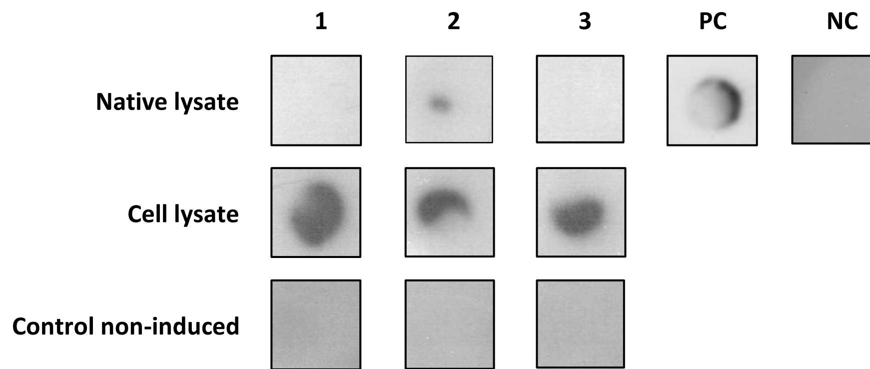


Fig.4.17: Testing of production of tVP2 in induced bacteria. Dot blot of bacterial lysates. Native lysate (1 – 3): native lysates of BL21 after induced expression of tVP2, Cell lysate (1 – 3): cell lysates of BL21 after induced expression of tVP2, Control non-induced (1 – 3): control non-induced cell lysates. Numbers 1 - 3 represent three parallel experiments. Primary antibody: commercial antibody against His-tag (dilution 1:100), secondary antibody: goat anti rabbit fused with HRP (dilution 1:1000). Film was exposed for 10 minutes.

4.3.3.1. Repeated electroporation

Due to problems with tVP2 production, a control electroporation of BL-21 bacteria was performed. Plasmid DNA from colony 11 (XL1 blue bacteria, verified earlier, see chapter 4.3.2., Fig. 4.15 and Fig. S1) was isolated from bacteria cultivated overnight and electroporated into the BL-21 bacteria. These were applied on agar plates with kanamycin and were cultivated overnight.

Three monocolonies were inoculated into 2xYT medium with kanamycin and were cultivated overnight. In the morning bacteria samples were diluted to OD_{600} = 0.1 and were cultivated until OD_{600} reached ~0.7. Then, T7 RNA polymerase expression was induced by addition of IPTG (final concentration 0.1 mM). Bacteria were cultivated at 28°C for 3 hours. Native and cell lysates were tested by dot blot (Fig. 4.18). Based on obtained results no expression occurred or the expression level was undetectably low. Optimization was applied using bacteria used in chapter 4.3.3. in order to increase protein expression.

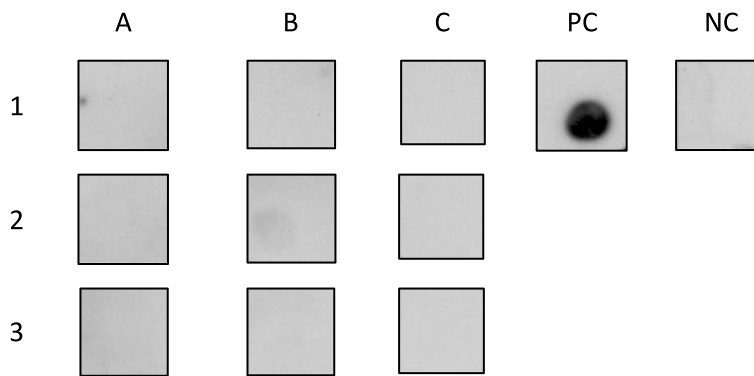


Fig.4.18: Production of tVP2 in bacteria after repeated electroporation. Dot blot from native lysates (column A), cell lysates (column B) and non-induced control cell lysates (column C) from three parallel experiments (1, 2, 3) were tested on dot blot. PC: Protein VP1of MPyV fused with CAP and His-tag was used as positive control (kindly provided by M. Fraiberk). Cell lysate of BL-21 bacteria with no extra plasmid was used as negative control (NC). Primary antibody: commercial antibody against His-tag (dilution 1:100). Secondary antibody: goat anti rabbit fused with HRP (1:1000). Film was exposed for 10 minutes.

4.3.3.2. Optimization of expression

Protein expression is a tricky procedure especially in case of protein with hydrophobic regions like tVP2. Optimization is often necessary to increase protein yield sufficiently. Based on results represented above, optimization was performed using BL-21 bacteria electroporated with plasmid isolated from colony 12 (XL1 blue).

4.3.3.2.1. Temperature optimization

The first condition to be optimized is usually the temperature in which bacteria are cultivated when expressing. Three temperatures were selected: 28°C, 20°C and 15°C and cultivation time was extended to overnight expression. Native and cell lysates (see chapters 3.2.5.1. and 3.2.5.2. for details of the procedure) were prepared and tested by dot blot (Fig. 4.18). Expression of tVP2 protein was detected in bacteria cultivated in 28°C, no expression was detectable in lower temperatures.

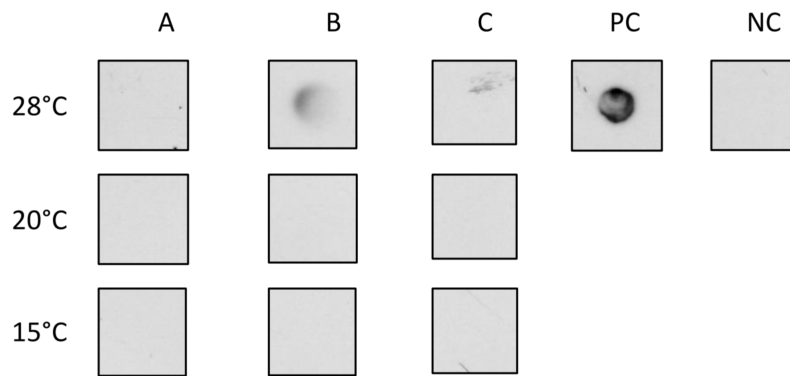


Fig.4.19: Production of tVP2 in bacteria under different cultivation temperatures. Dot blot from native lysates (column A), cell lysates (column B) and non-induced control cell lysates (column C) from three parallel experiments (28°C, 20°C, 15°C) were tested on dot blot. PC: A protein of MPyV VP1 fused with CAP and His-tag was used as positive control (kindly provided by M. Fraiberk). BL-21 bacteria with no extra plasmid were used as negative control (NC). Primary antibody: polyclonal antibody against His-tag (dilution 1:100). Secondary antibody: goat anti rabbit fused with HRP (dilution 1:1000). Film was exposed for 10 minutes.

4.3.3.2.2. IPTG concentration balancing

To improve the expression of tVP2 protein, influence of different concentrations of IPTG was tested. Presence of IPTG is the essential condition for protein expression in T7 RNA polymerase system. Without IPTG, T7 RNA polymerase expression is not induced and recombinant plasmid DNA cannot be transcribed to mRNA. Due to the fact that this condition is so crucial, higher IPTG concentration (than initially used 0.1mM) was tested.

Concentration of 1mM IPTG (10-times higher than in previous experiment) was applied on bacteria and expression proceeded for 2 hours at 28°C. Bacterial culture OD_{600} was measured at the end of the experiment to rougly monitor bacterial growth ability after inducting expression (see chapter 3.2.4.5. for details of the procedure). Bacterial growth ability after induction with 1mM IPTG was significantly reduced in comparison to previous experiments. Doubling times were determined from measured OD_{600} and extension of doubling time for induced bacteria in comparison to control bacteria is shown in Tab. 4.1 in percents. OD_{600} graph is not linear over 1, but in this case, numbers were used solely for comparison of the two samples (measured under identical conditions), however, results are only estimative and should be considered as such. Native and cell lysates were prepared and tested by dot blot (Fig. 4.20).

	15°C, overnight, **0.1mM IPTG**	28°C, 3 hours, **0.1mM IPTG**	28°C, 2 hours, **1mM IPTG**
START	0.891	0.689	0.422
END_{ind}	2.267	1.604	0.949
$END_{control}$	2.331	1.666	1.416
doubling time exntension (in induced compared to control)	**2.83%**	**4%**	**47.22%**

Tab.4.1: OD_{600} of bacterial cultures was measured in the beginning (START) of induced protein expression and when the expression was ended in induced bacteria (END_{ind}) and non-induced control bacteria ($END_{control}$). First column: bacteria expressing overnight (15 hours) at 15°C induced with 0.1mM IPTG (see chapter 4.3.3.1.1.). Second column: bacteria expressing for 3 hours at 28°C induced with 0.1mM IPTG (see chapter 4.3.3.1.2.). Third column: bacteria expressing for 2 hours at 28°C induced with 1mM IPTG. The difference between END_{ind} and $END_{control}$ is significantly higher in bacteria induced with higher concentration of IPTG as represented by extension of bacterial doubling time shown in percents (counted for each experiment). Results should be considered as estimative since OD_{600} graph is not linear over 1.

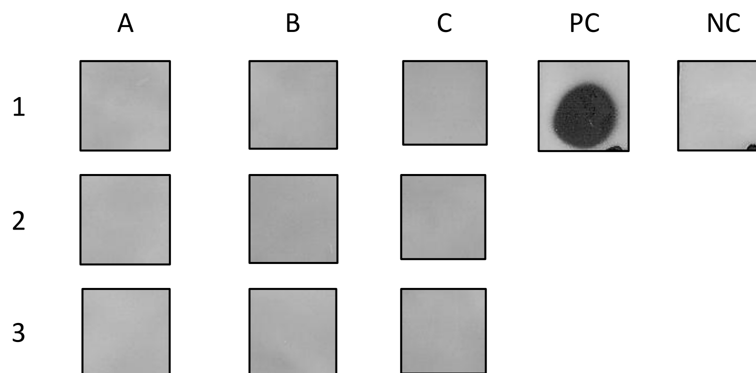


Fig.4.20: Production of tVP2 in bacteria under different concentration of IPTG. Dot blot from native lysates (column A), cell lysates (column B) and non-induced control cell lysates (column C) from three parallel experiments (1, 2, 3) were tested on dot blot. PC: VP1 protein of MPyV fused with CAP and His-tag was used as positive control (kindly provided by M. Fraiberk). Cell lysate of BL-21 bacteria with no extra plasmid was used as negative control (NC). Primary antibody: commercial antibody against His-tag (dilution 1:100). Secondary antibody: goat anti rabbit fused with HRP (dilution 1:1000). Film was exposed for 11 minutes.

4.3.3.1.4. Alternative protocol

When we were not able to produce tVP2 even under different experimental conditions, an alternative protocol was applied. This alternative protocol had been successfully used earlier in our

laboratory, in a similar case of protein production failure (M. Fraiberk, personal communication). The principal difference between the two protocols was earlier IPTG induction. Bacterial culture was induced when OD_{600} was 0.1 instead of 0.7 (see chapter 3.2.4.6.3. for details of the procedure). After induction, bacteria were cultivated overnight at 28°C. Sample bacteria and non-induced control were centrifuged, cell lysates were prepared and tested by dot blot (Fig.4.21).

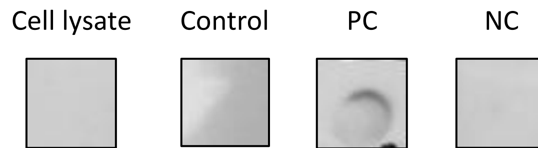


Fig.4.21: Production of tVP2 in bacteria after overnight cultivation. Dot blot from cell lysate and non-induced control cell lysates (control) were tested on dot blot. PC: Protein of MPyV VP1 fused with CAP and His-tag was used as positive control (kindly provided by M. Fraiberk). Cell lysate of BL-21 bacteria with no extra plasmid was used as negative control (NC). Primary antibody: commercial antibody against His-tag (dilution 1:100). Secondary antibody: goat anti rabbit fused with HRP (dilution 1:1000). Film was exposed for 15 minutes.

4.3.3.2. Detection of protein integrity

Detection of produced protein was always based on His-tag detection. His-tag is expressed on C-terminus of the protein in the setting of pET29b plasmid (Fig. 4.13) and therefore it is expressed last. In case of translation or transcription process failure or protein degradation, His-tag could not be present, even if N-terminal part of the protein could be produced or some smaller degradation products could be present. In order to detect whether translation (transcription) failure or protein degradation could be the problem in this case, detection of an epitope close to protein N-terminus was necessary. Thanks to the presence of an N-terminal tag (S-tag, Fig. 4.13), S-tag detection could be performed in bacterial lysates.

Expression under various conditions was performed and tested by Western blot. Verified DNA from two bacterial colonies – 11 and 12 (see chapter 4.3.2.) was electroporated into BL-21 bacteria. Bacteria were cultivated overnight. Two bacterial monocolonies from each electroporation were tested for tVP2 insert by colony PCR (Fig.4.22) (see chapter 3.2.3.9.1.).

Expression of protein was induced under conditions described in chapters 4.3.3. (28°C, 3 hours, 0.1mM IPTG), 4.3.3.1.1. (20°C and 15°C, overnight, 0.1mM IPTG), 4.3.3.1.3. (28°C, 3 hours, 1mM IPTG) and 4.3.3.1.4. (alternative protocol, 28°C, 3 hours, 0.1mM IPTG). After expression bacteria were collected by centrifugation and cell lysates were prepared and tested by Western blot (Fig. 4.23). In case of positive signal, native lysates were also tested (Fig. 4.24).

Based on obtained results, colony 11a and 28°C, 3 hours, 1mM IPTG were chosen as optimal for production. Bacteria were cultivated overnight and expression procedure was performed as described above. Native and cell lysates were prepared and tested on SDS PAGE (Fig. 4.24) and Western blot (Fig. 4.25). Gel after the blotting procedure was also stained in CBBG solution, which revealed serious blotting problems in a band roughly corresponding to the expressed protein (Fig. 4.26).

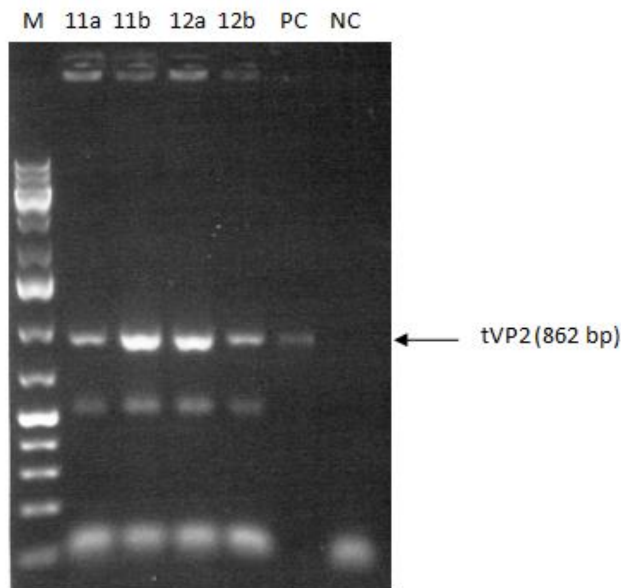


Fig. 4.22: Agarose electrophoresis from colony PCR verification of tVP2 insert in bacterial monocolonies. Two bacterial monocolonies were chosen after electroporation with DNA from colonies 11 and 12 (11a, 11b, 12a, 12b). M: marker, PC: tVP2 DNA (862 bp) prepared by PCR and used for cloning, NC: PCR reaction with no template (no bacterial lysate). All colonies included tVP2 DNA, smaller fragment was produced probably as a result of unspecific primers annealing.

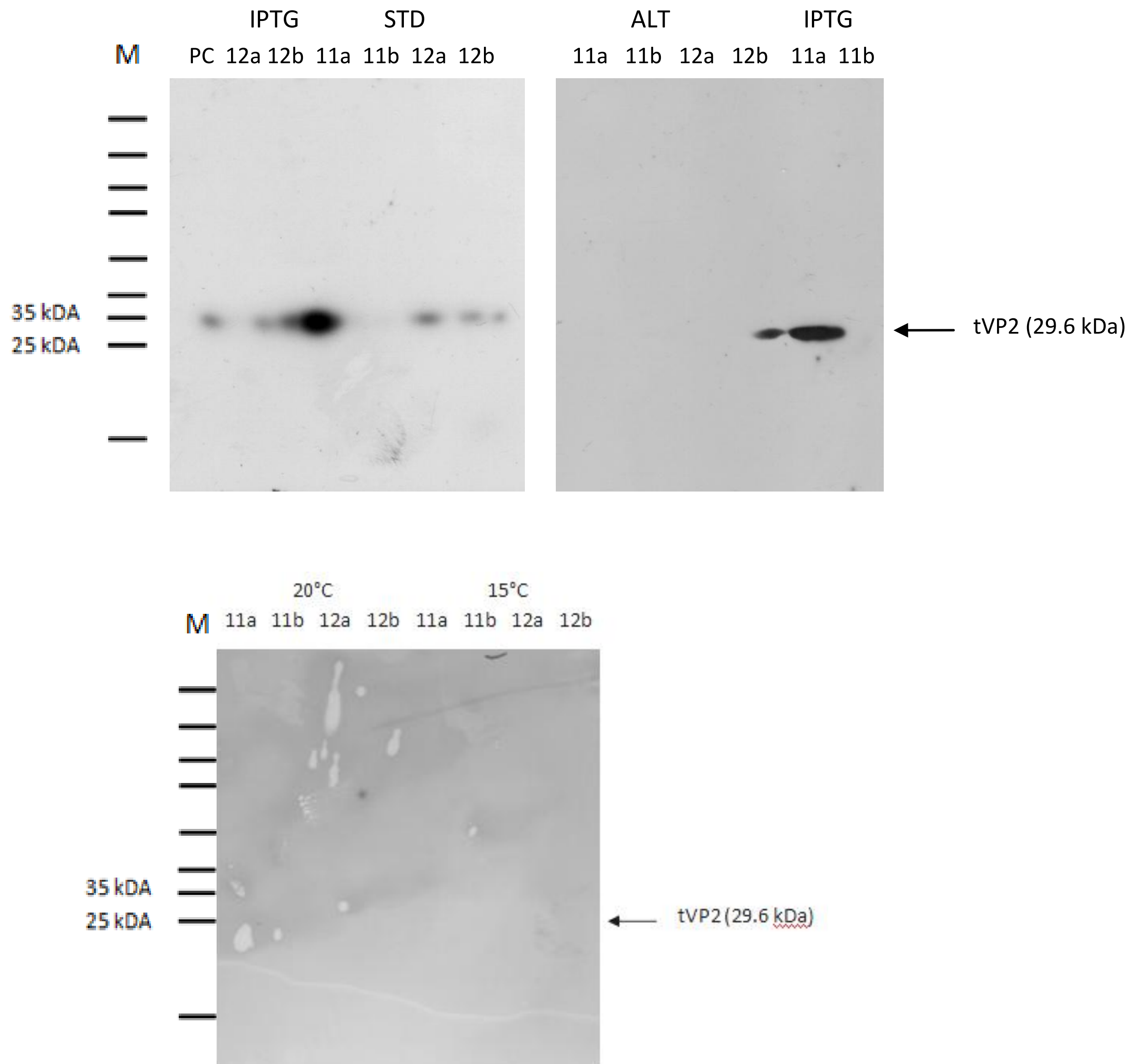


Fig. 4.23: Western blot analyses of cell lysates after induced expression of tVP2. Expression in colonies 11a, 11b, 12a and 12b using standard protocol (28°C, 3 hours, 0.1mM IPTG; STD), alternative protocol (28°C, 3 hours, 0.1mM IPTG; ALT), higher IPTG (28°C, 3hours, 1mM IPTG; IPTG) and lower temperatures (20°C and 15°C, overnight, 0.1 mM IPTG; 20°C, 15°C respectively).VP2unique (unique part of VP2 BKV with His-tag and S-tag, prepared by M. Verdánová) was used as positive control (PC). Bacteria BL-21 with no electroporated plasmid (cell lysate) were used as negative control (NC). Membrane was stained with S-tag antibody (diluted 1:2000) and goat anti-mouse conjugated with HRP was used as secondary antibody (diluted 1:1000). Film was exposed for 15 minutes.

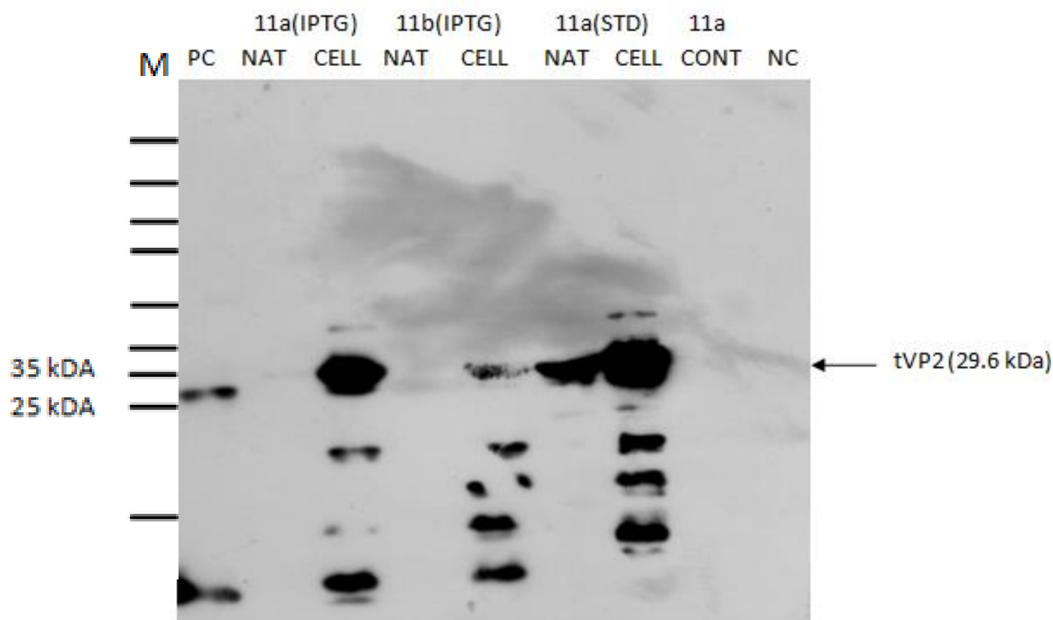


Fig.4.24: Western blot analyses of native (NAT) and cell (CELL) lysates after induced expression of tVP2 and lysates from non-induced control bacteria (CONT). Expression was performed in colonies 11a and 11b using standard protocol with higher IPTG concentration (28°C, 3 hours,1mM IPTG).VP2unique (unique part of VP2 BKV with His-tag and S-tag, prepared by M. Verdánová) was used as positive control (PC). Bacteria BL-21 with no electroporated plasmid (cell lysate) were used as negative control (NC). Membrane was stained with S-tag antibody (diluted 1:1000) and goat anti-mouse conjugated with HRP was used as secondary antibody (diluted 1:1000). Film was exposed for 5 minutes.

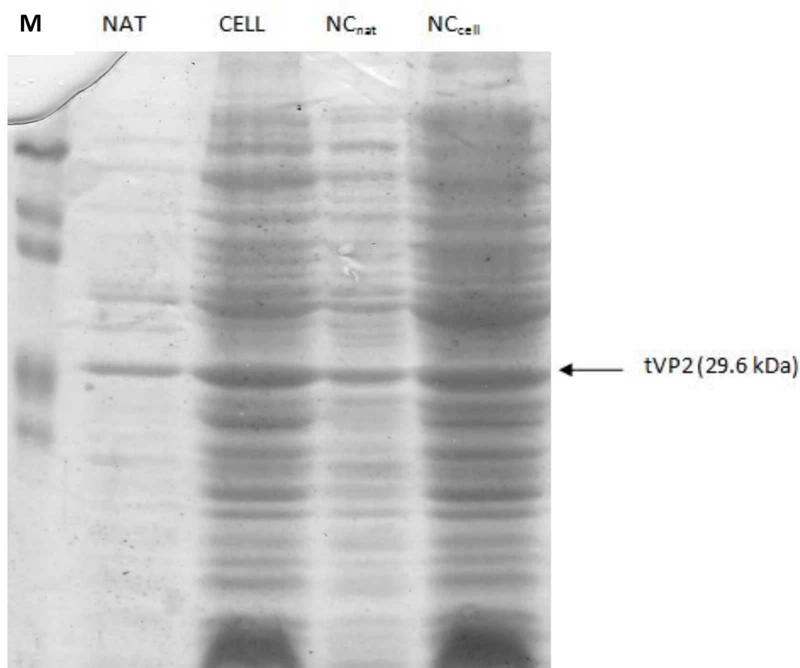


Fig.4.25: SDS PAGE of native (NAT) and cell (CELL) lysates after induced expression of tVP2. Expression was performed using standard protocol with higher IPTG concentration (28°C, 3 hours,1mM IPTG). Bacteria BL-21 with no electroporated plasmid (native and cell lysates) were used as negative controls (NC_{nat} and NC_{cell}, respectively). M: marker. Band in cell lysate marked as tVP2 should include the expressed protein as shown on Western blot.

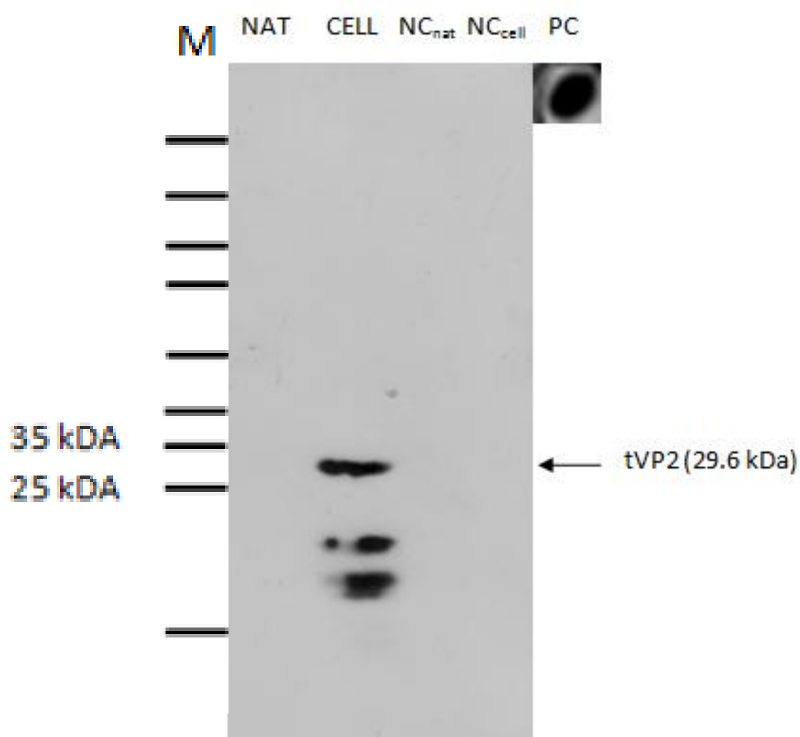


Fig.4.26: Western blot analyses of native (NAT) and cell (CELL) lysates after induced expression of tVP2. Expression was performed using standard protocol with higher IPTG concentration (28°C, 3 hours,1mM IPTG).VP2unique (unique part of VP2 BKV with His-tag and S-tag, prepared by M. Verdánová) was used as positive control (PC) in form of a dot on a separate membrane (stained and treated identically). Bacteria BL-21 with no electroporated plasmid (native and cell lysates) were used as negative controls (NC_{nat} and NC_{cell}, respectively). Membrane was stained with S-tag antibody (diluted 1:1000) and goat anti-mouse conjugated with HRP was used as secondary antibody (diluted 1:1000). Film was exposed for 10 minutes. Bands of smaller molecular weight probably correspond to degradation products.

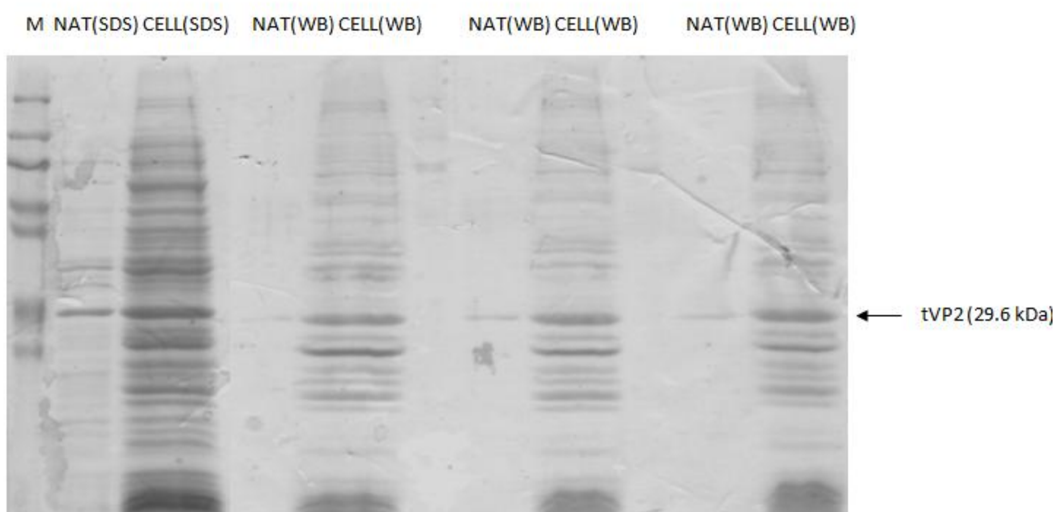


Fig. 4.27: Blotting efficiency is significantly diminished in a band corresponding to recombinant tVP2 protein. SDS PAGE of native (NAT) and cell (CELL) lysates after induced expression of tVP2, shown are four sets of the same samples. Gel was stained right after SDS PAGE (SDS) or after Western blotting (WB). M: marker.

4.3.3.3. Protein purification on HIS-Select® Nickel Affinity Gel

Since the data obtained from S-tag detection implied that at least small amount of tVP2 could be produced in correct length (including His-tag), an attempt to purify protein was performed. Native lysate of bacteria was applied on HIS-Select® Nickel Affinity Gel and incubated for 1 hour. Samples from flow through after lysate application, washing, elution and the used gel were tested on SDS PAGE (Fig. 4.28). Western blot was performed from the samples as well, but no signal was detected probably due to very low amount of protein in combination with low blotting efficiency (data not shown). Polyacrylamide gel was stained after blotting and the staining confirmed the blotting complications described in chapter 4.3.3.2.

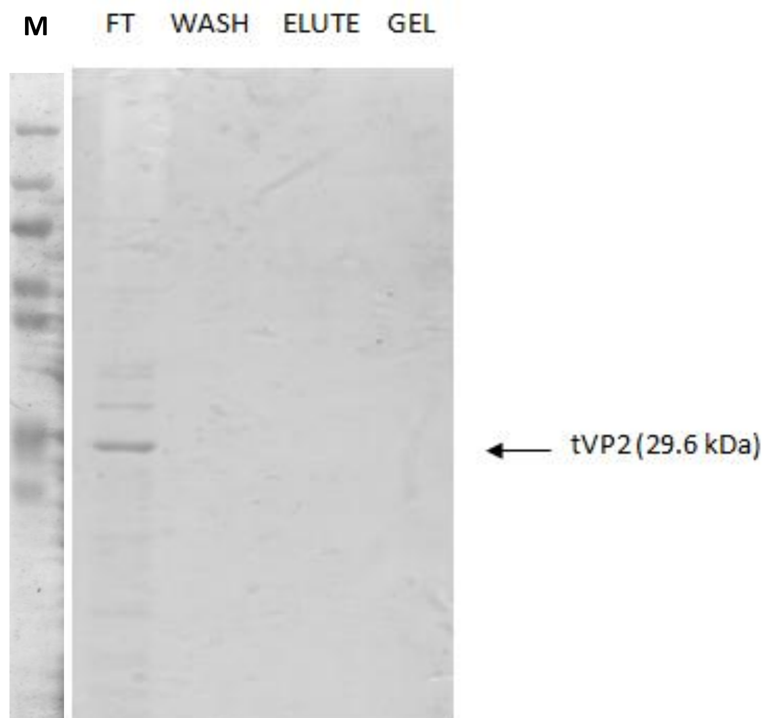


Fig.4.28: The results of affinity purification of tVP2 protein. SDS PAGE of samples obtained during recombinant protein purification. Sample that was obtained after incubation of bacterial lysate with the gel was marked as flow through (**FT**), supernatant after washing the gel was marked as **WASH**. Sample obtained after elution buffer treatment was marked as **ELUTE**. The pelleted gel was also tested for residual protein content (**GEL**). **M**: marker

4.4. Preparation of recombinant baculoviral expression system for production of MCPyV virus-like particles (VLPs)

Virus-like particles (VLPs) are artificially expressed viral capsids. Polyomaviral capsids consist of three structural proteins, major VP1 and minor proteins VP2 and VP3. Major protein VP1 is able to form a capsid by itself when expressed in an *in vitro* expression system. When VP2 is coexpressed, it

incorporates into the VLPs (see chapter 2.1.3. for more information). A recombinant baculoviral system was used for production of MCPyV VP1/VP2 VLPs, while this system works well in our laboratory for other polyomavirus structural proteins and VLPs preparation.

4.4.1. Preparation of recombinant plasmid

Plasmid pFastBac^{TM} Dual was used to clone DNA for MCPyV VP1 and VP2 (Fig. 4.29, see chapter 3.1.6. for details of the procedure). PCR was used for the preparation of VP1 and VP2 DNA for insertion. Plasmids pwm (VP1) and ph2m (VP2) from Addgene served as templates. Primers for PCR were designed with restriction sites for restriction nucleases NheI and XhoI (VP1) or SalI and PstI (VP2) (see chapter 3.1.7. for primers sequences).

Plasmid pFastBac^{TM} Dual was isolated by QIAprep Spin Miniprep Kit (Qiagen) from bacteria cultivated overnight. Concentration was measured by Nanodrop, being 95.5 ng/µl. PCR product for VP1 insert (Fig. 4.30) was prepared by PCR (see chapter 3.2.3.8. for details of the procedure), cut by NheI and XhoI restriction endonucleases. The same restriction cleavage was applied to the plasmid. Successful cleavage of VP1 DNA was verified by ligation ladder (see chapter 3.2.3.7.1. for details of the procedure). Plasmid was dephosporylated with alkaline phosphatase to avoid further religation. After cleavage, DNA was purified by phenol-chloroform method. Ligation mix was prepared in molar ratio 3:1 (insert: plasmid), ligation reaction ran overnight at 22° C.

Ligation mix and a negative control (ligation mix with no insert) were electroporated into XL-1 blue bacteria. Bacteria were applied on agar plates with ampicilin in several dilutions. Plates with bacteria electroporated with negative control contained 10 times lower amount of colonies than the same dilution of sample bacteria. Bacterial monocolonies presumably containing our recombinant plasmid were tested for its presence by minipreparation of DNA and further cleavage with EcoRI restriction endonuclease. Restriction sites for EcoRI can be found in DNA sequence for VP1 and in plasmid multiple cloning site under polyhedrin promoter (P_{PH}, Fig. 4.29). Cleavage of pFastBac Dual containing VP2 sequence should revealed two segments – 1 kbp and 8 kbp. Out of twelve colonies tested, nine included the correct plasmid (Fig. 4.31). One of these nine colonies (colony 11) was sequenced (see supplement for sequencing data, Fig. S2) to verify correct insertion.

Similar process to the one described above was applied when inserting DNA for protein VP2 into the plasmid. Plasmid pFastBac^{TM} Dual with inserted DNA for VP1 was isolated by QIAprep Spin Miniprep Kit (Qiagen) from bacteria cultivated overnight. Concentration of plasmid was 73.2 ng / µl. SalI and PstI restriction endonucleases were used for cleavage of plasmid and insert (VP2 DNA prepared by PCR, Fig. 4.32). Successful cleavage of VP2 DNA was verified by ligation ladder (see chapter 3.2.3.7.1.

for details of the procedure). Plasmid was dephosporylated with alkaline phosphatase. After cleavage, DNA was purified by phenol-chloroform method. Ligation mix was prepared in molar ratio 3:1 (insert:plasmid), ligation reaction ran overnight at 22° C. Ligation mix and a negative control (ligation mix with no insert) were electroporated into XL1 blue bacteria. Bacteria were applied on agar plates with ampicilin in several dilutions. Plates with bacteria electroporated with negative control contained 10 times lower amount of colonies than the same dilution of sample bacteria. Bacterial monocolonies presumably containing the recombinant plasmid were tested for its presence by minipreparation of DNA and correct insertion was verified using XhoI restrictase. Restriction sites for XhoI can be found in DNA sequence for VP2 and in plasmid multiple cloning site under p10 promoter (P_{p10}, Fig. 4.29). Cleavage of pFastBac Dual containing VP1 and VP2 sequences should reveal fragments of 400 bp and 10,600 bp. Out of 18 colonies tested, 14 were positive (Fig. 4.33). One of these bacterial colonies (Colony 12) was sequenced to verify insert sequence (see supplement for sequencing data, Fig. S3).

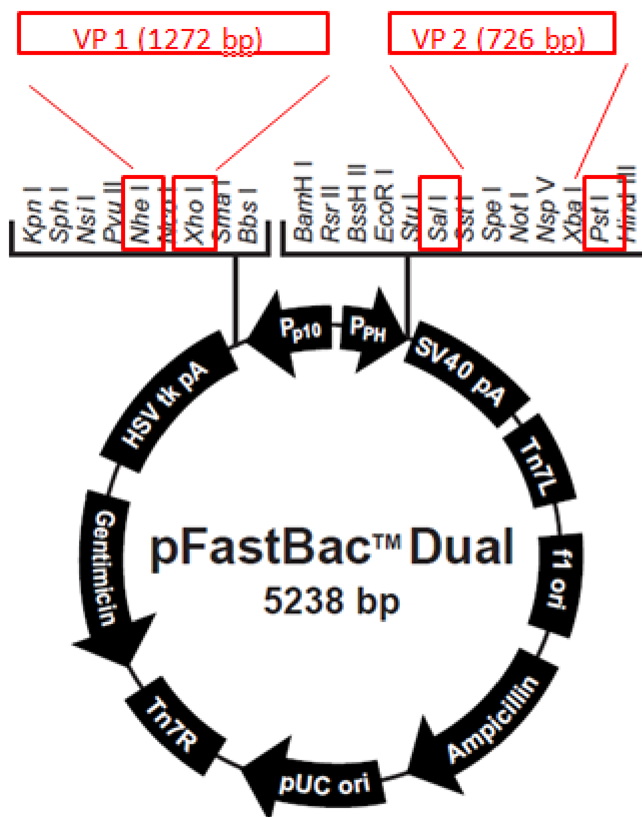


Fig. 4.29: Scheme of plasmid pFastBac^{TM} Dual, including multiple cloning sites under polyhedrin (P_{PH}) and p10 (P_{p10}) promoters with intended insert sequences (VP2 and VP1 DNA, respectively), ampicilin and gentamicin resistance gene, recombination sites Tn7R and Tn7L.

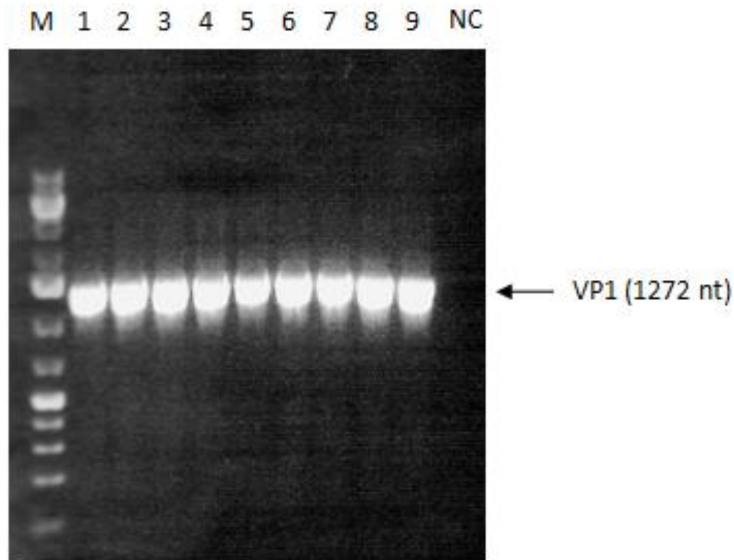


Fig. 4.30: **Agarose electrophoresis of PCR product – VP1 DNA;** M-marker, 1-9 PCR products, NC- negative control (PCR mix with no template). Samples were prepared while testing appropriate annealing temperature (annealing temperatures from 60.9°C in sample 1 to 71°C in sample 9). Yield in all samples was equally specific and in equal amount. All samples were combined and used for cloning.

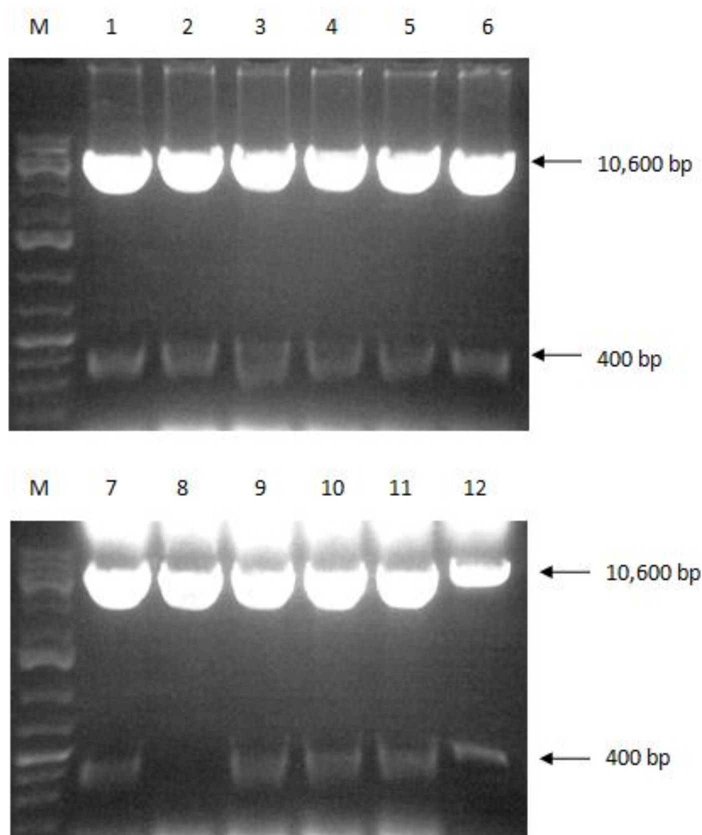


Fig. 4.31: **Restriction verification of pFastBac Dual plasmid with MCPyV VP1 sequention.** Lines 1-12: Plasmid DNA isolated by minipreparation from different bacterial monocolonies (see chapter 3.2.3.2.1. for details of the procedure) and cut by EcoRI. Plasmids with correct VP1 insertion revealed two segments (1 kbp and 8 kbp) after cleavage. Plasmid pFastBac^{TM} Dual with no insert was used as negative control (NC).

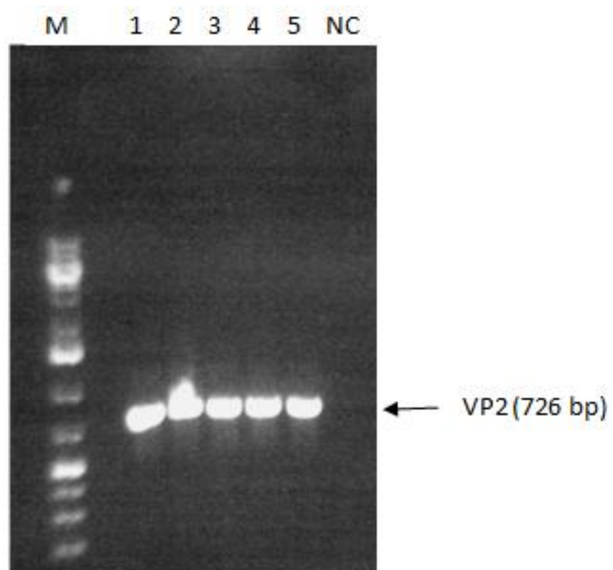


Fig. 4.32: **Agarose electrophoresis of PCR product – VP2 DNA;** M: marker, 1-5 PCR products, NC: negative control (PCR mix with no template). Samples were prepared as described in 3.2.3.9., annealing temperature was set based on primer testing as 68.6°C. All samples were combined and used for cloning.

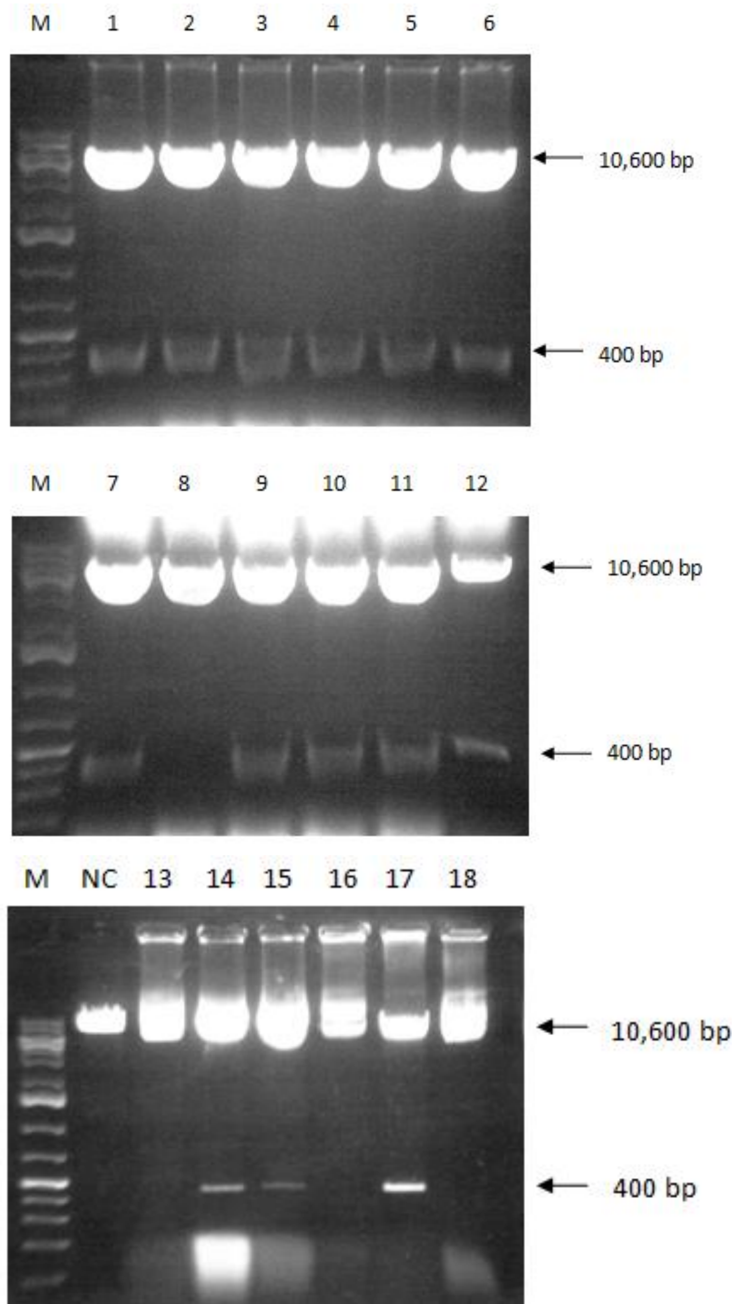


Fig. 4.33: **Restriction verification of pFastBac™ Dual with VP1 and VP2 DNA.** Lines 1-18: Plasmid DNA isolated from different monocolonies by minipreparation (see chapter 3.2.3.2.1.) and cut by XhoI. Plasmid with correct insertion revealed two segments (400 bp and 10,600 bp) after cleavage. NC: plasmid pFastBac™ Dual with only VP1 DNA.

4.4.2. Preparation and verification of recombinant bacmid

Recombinant baculoviral genome was prepared in the Bac-to-bac system, by recombination between recombinant pFastBac™ Dual with MCPyV VP1 and VP2 plasmid and a pFastBac™ bacmid in DH10Bac™ bacteria (*E. Coli*). After electroporation of pFastBac Dual with MCPyV VP1/VP2 to bacteria, these were applied on agar plates with three selection antibiotics (tetracycline, kanamycin

and gentamicin) and with components for white-blue selection (X-gal and IPTG). Colonies with successful recombination cannot produce blue product (X-gal cleaved by LacZα) due to disruption of LacZα sequence by insert. Three days after electroporation, three white colonies (A, B, C) were inoculated into cultivation medium with selecting antibiotics. Bacmids were isolated successfully, in duplicates (further marked with numbers 1 and 2) (see chapter 3.2.3.2.2. for details of the procedure, Fig. 4.34).

Inserted sequences were tested by colony PCR, verifying the presence of VP1 and VP2 DNA (Fig. 4.35). Concentration of DNA was measured on nanodrop, concentration was 38.7 ng/µl and 29.1 ng/µl in samples A1 and A2, respectively; 43.1 ng/µl and 31.5 ng/µl in samples B1 and B2, respectively; and 64.4 ng/µl and 31 ng/µl in samples C1 and C2, respectively. Inserts in bacmids B (sample B1) and C (sample C1) were chosen for sequencing verification (see supplement for sequencing data, Fig.S4). Sample B1 was chosen to verify that the two bands visible on agarose gel (Fig. 4.33) represent two isoforms of the same bacmid. Sample C1 was chosen as the most reliable sample in terms of DNA concentration and appearance on agarose gel. Samples A1 and A2 could not be chosen for sequencing due to low DNA concentration. Bacmids B and C were isolated from bacteria cultivated overnight, in duplicates (B, B*, C, C*) and the four samples were used for transfection of insect cells Sf9.

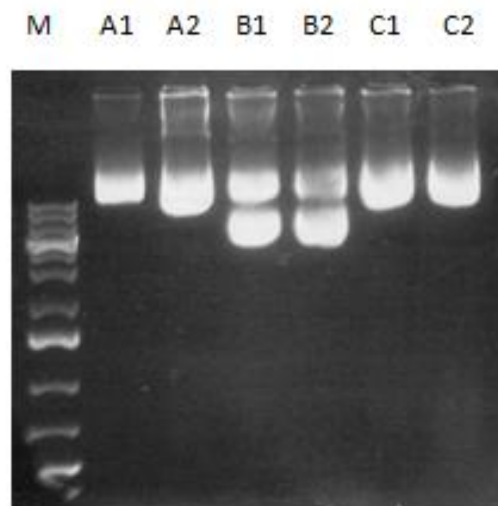


Fig. 4.34:**Agarose electrophoresis of recombinant bacmids after isolation from bacterial monocolonies.** High molecular DNA (136 kb) is visible in all isolations. Sample B contains probably two bacmid isoforms. Isolation was performed in duplicates to insure successful isolation.

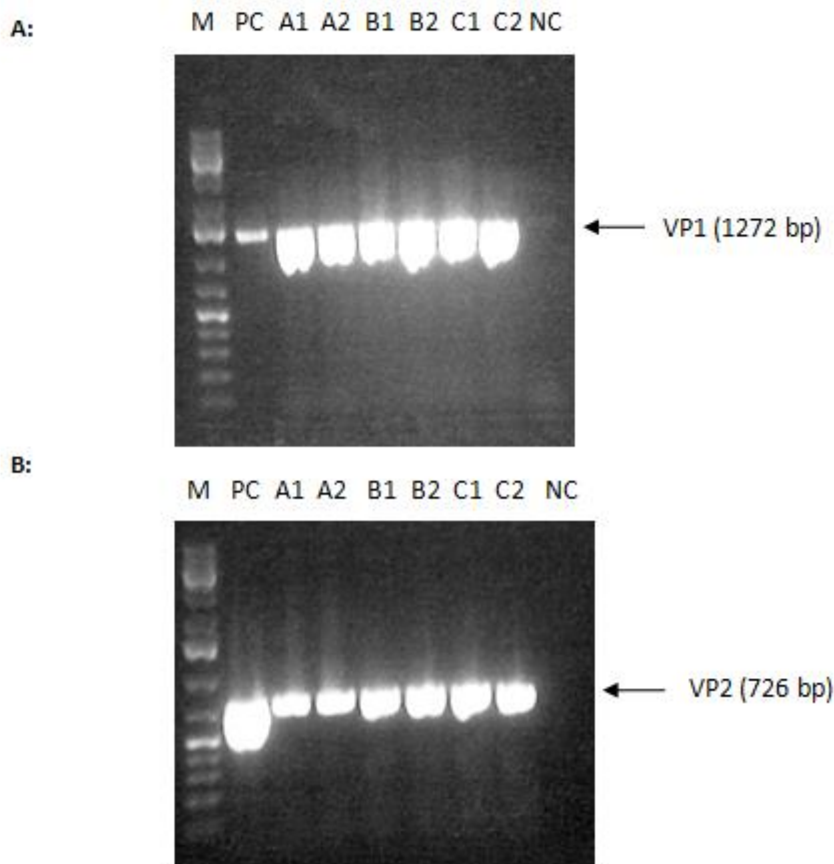


Fig. 4.35:**Agarose electrophoresis of PCR products for VP1 (A) and VP2 (B) inserts in recombinant bacmids.** Bacmids were used as templates for PCR reactions. Every bacmid contained both inserts. M: marker, PC: DNA VP1 or VP2 used for insertion into plasmid pFastBac™ Dual. NC: PCR mix with no template (no bacmid).

4.4.3. Transfection of insect cells Sf9

Sf9 cells in medium supplemented with 10% FBS (no antibiotics) were distributed into 6-well plate ($9 \cdot 10^5$ cells per well). Before transfection, medium was replaced with one supplemented with 1.5% FBS (no antibiotics) and mixture of cellfectin preincubated with bacmid DNA was applied on cells (see chapter 3.2.7.1 for details of the procedure).

4.4.4. Baculoviral stock purification and testing

Three days after transfection, media with produced recombinant baculoviruses were collected. Cells were lysed in RIPA buffer (see chapter 3.2.5.3. for details of the procedure), lysates were tested for the presence of VP1 on dot blot (Fig. 4.36). The presence of VP2 was not tested, due to absence of suitable antibody. Inoculum B* was further purified by plaque assay (see chapter 3.2.7.2 for details of the procedure).

Four plaques were used for infection of Sf9 cells. Three days after infection, media was collected and cells were lysed in RIPA buffer and tested by dot blot (Fig. 4.37). Signal on dot blot from plaque infection lysates is incomparably lower than in previous dot blot since a single plaque includes relatively low amount of baculovirus (each plaque is derived from a single viral particle, see chapter 3.2.7.2.) and so baculoviral inoculum obtained after this type of infection needs to be enriched. Medium from infection with plaque 2 was further used as inoculum for repeated infections of Sf9 cells to enrich the volume and viral concentration.

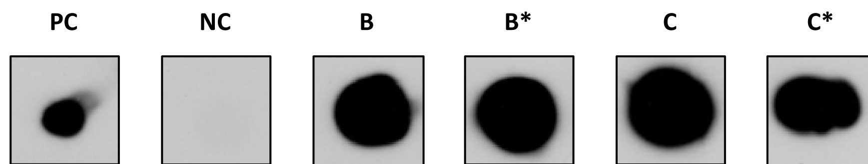


Fig. 4.36: **Testing of MCPyV VP1 protein presence in transfected Sf9 cells by dot blot.** Cells were lysed in RIPA buffer, three days after transfection. MCPyV VP1 VLPs (prepared and kindly provided by P. Sauerová) were used as positive control (PC). Mock transfected Sf9 cells were used as negative control (NC). Polyclonal antibody against VP1 of MCPyV was used as primary antibody. Goat antibody conjugated with HRP against mouse immunoglobulins was used as secondary antibody (dilution 1:1000). Film was exposed for 6 minutes.

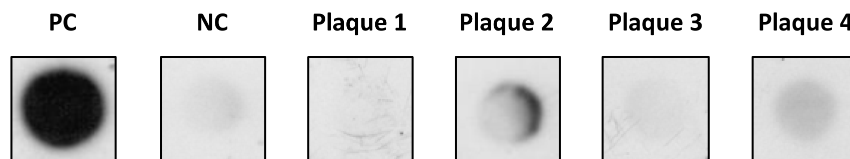


Fig. 4.37: **Testing of VP1 protein presence in infected Sf9 cells by dobtlot.** Cells were infected with baculoviruses obtained from four different plaques. Cells were lysed in RIPA buffer, three days after infection. Sf9 cell lysate from transfection B* were used as positive control (PC). Mock infected Sf9 cells were used as negative control (NC). Polyclonal antibody against VP1 of MCPyV was used as primary antibody. Goat antibody conjugated with HRP against mouse immunoglobulins was used as secondary antibody (dilution 1:1000). Film was exposed for 15 minutes.

4.4.5. Baculoviral inoculum testing

The selected recombinant baculovirus was propagated in Sf9 cells. After set of propagation steps, another plaque assay was performed to determine virus concentration (see chapter 3.2.7.2. for details of the procedure). Concentration was $5.4 \cdot 10^7$ pfu/ml. Production of MCPyV VP1 protein was tested by dot blot (Fig. 4.38), SDS PAGE (Fig. 4.39), western blot (Fig. 4.40) and immunofluorescence assay (Fig. 4.41).

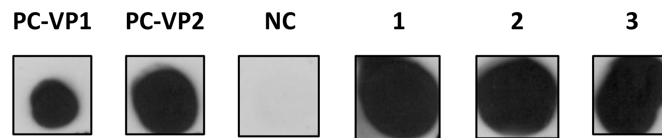


Fig. 4.38: **Testing of VP1 protein presence in infected Sf9 cells by dot blot.** Cells were infected with recombinant baculovirus expressing proteins VP1 and VP2 of MCPyV. Cells were lysed in RIPA buffer, three days after infection. VLPs consisting of only VP1 protein (prepared and kindly provided by P. Sauerová) and Sf9 cell lysate from transfection B* were used as positive controls (PC-VP1 and PC-VP2, respectively). Mock infected Sf9 cells were used as negative control (NC). Samples 1, 2 and 3 are three parallel experiments (infected cells from one Ø 6 cm each experiment); 3 µl of lysates was applied on membrane. Polyclonal antibody against VP1 of MCPyV was used as primary antibody. Goat antibody conjugated with HRP against mouse immunoglobulins was used as secondary antibody (dilution 1:1000). Film was exposed for 3 minutes.

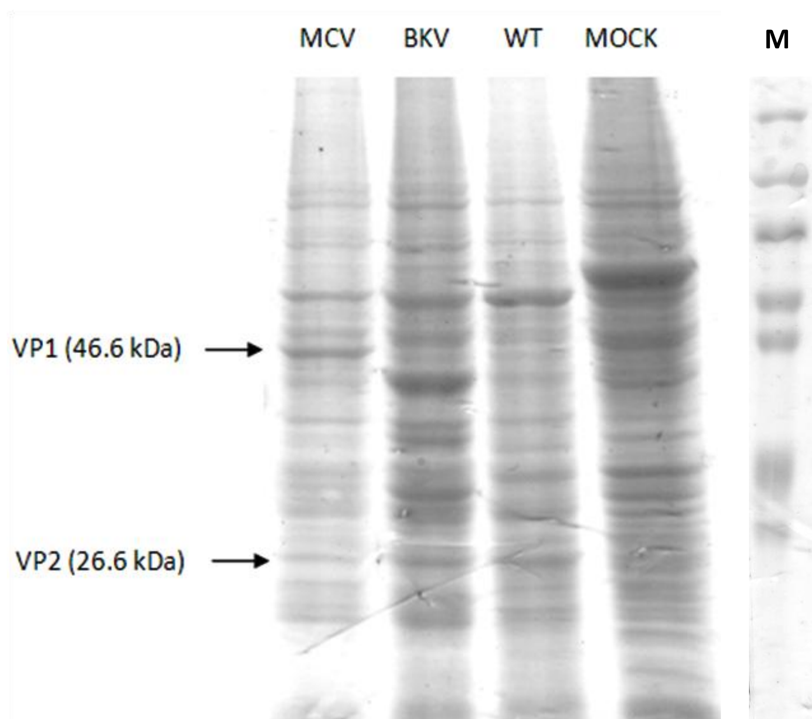


Fig. 4.39: SDS PAGE of lysates prepared from Sf9 cell infected by various recombinant baculoviruses. Sf9 cells were infected with a baculovirus producing MCPyV-VP1/VP2 proteins (MCV), BKV-VP1/VP2 proteins (BKV), wildtype baculovirus (WT) or mock-infected (MOCK). Cells were collected 4 d.p.i. and RIPA-buffer lysates were prepared. Production of recombinant proteins VP1 (46.6 kDa) and VP2 (26.6 kDa) of MCPyV can be seen in MCPyV line. Expression level of recombinant proteins is comparable with another recombinant baculovirus (line BKV). M: marker

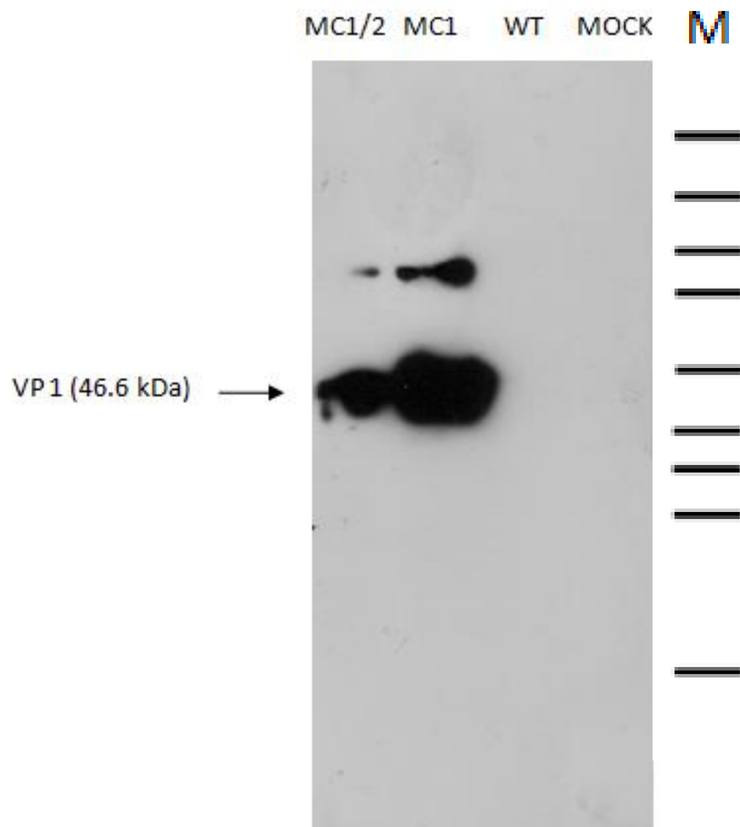


Fig. 4.40: **Testing of VP1 protein presence in infected cells by Western blot.** Cells were infected with recombinant baculovirus producing VP1 and VP2 of MCPyV (MC1/2) and VP1 of MCPyV (MC1). Cells infected with wild type baculovirus (WT) and mock infected cells (MOCK) were used as negative controls. Cells were lysed in RIPA buffer, three days after infection. Mouse serum against VP1 of MCPyV was used as primary antibody (dilution 1:1000). Goat antibody conjugated with HRP against mouse immunoglobulins was used as secondary antibody (dilution 1:1000). Film was exposed for 10 minutes.

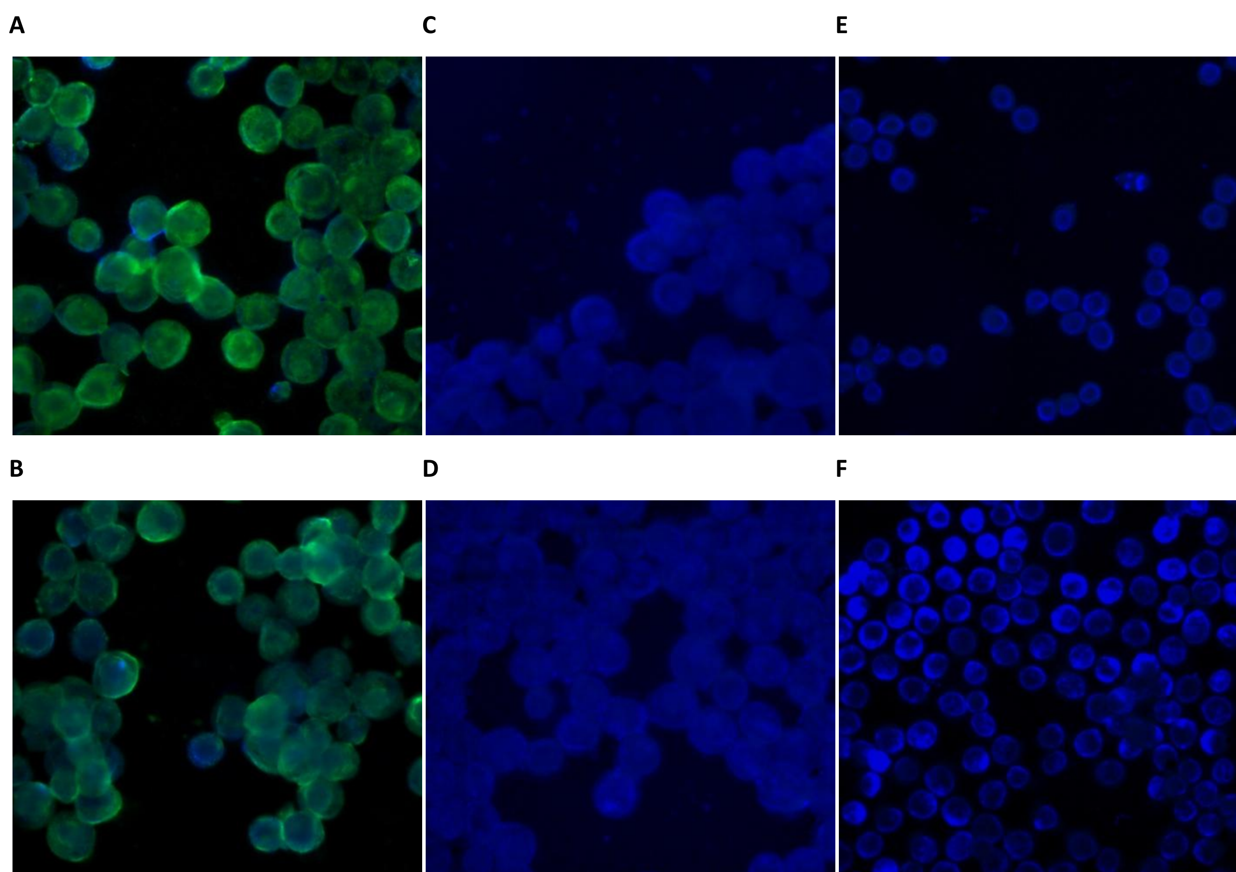


Fig. 4.41: **Testing of VP1 protein presence in infected Sf9 cells by immunofluorescent staining.** Cells were infected with baculovirus expressing VP1 and VP2 proteins of MCPyV (a, b, MOI = 7), cells were fixed 4 d.p.i. in methanol-acetone (see chapter 3.2.1.5. for details of the procedure). Cells infected with wildtype baculovirus (c,d) and mock infected Sf9 cells (e,f) were used as negative controls. Polyclonal antibody against VP1 of MCPyV was used as primary antibody. Goat antibody conjugated with Alexa fluor 488 against mouse immunoglobulins was used as secondary antibody (dilution 1:1000). All pictures were taken with constant exposition (65 ms) chosen to eliminate unspecific signal in negative controls. Fields were magnified 40 times.

4.4.6. VLPs production and purification

4.4.6.1. Infection and cells collection

The verified recombinant baculovirus producing proteins VP1 and VP2 of MCPyV was used for infection of 20 plates, Ø 10 cm, with Sf9 cells. Cells were collected 4 d.p.i., washed in PBS and stored in -20°C for several days (dry pellets). Cells were thawed and twice sonicated in 1 ml of B buffer in 30 second long periods (see chapter 3.2.6. for details of the procedure). Supernatant containing VLPs was collected after centrifugation (6000g, 10 min, refrigerated centrifuge).

4.4.6.2. Ultracentrifugation

Collected supernatant was transferred into ultracentrifugation tubes and under-layered with 10% sucrose solution (a sucrose cushion). Sucrose cushion ultracentrifugation was applied in order to get rid of low molecular weight molecules. Ultracentrifugation in SW28 rotor at 25,000 rpm ran for three hours. Supernatant was removed and pellets slowly dissolved in B buffer.

Dissolved pellets were homogenized in Potter homogenizer and applied on top of caesium chloride solution in two ultracentrifugation tubes. Isopycnic centrifugation was performed in SW41 rotor at 35,000 rpm for 24 hours. Gradients 1 and 2 (formed during ultracentrifugation) were separated into fractions using peristaltic pump, yielding 14 and 12 fractions, respectively.

Presence of protein VP1 in fractions was tested by dot blot (Fig. 4.42), VLPs were found mainly in the upper fractions of the gradient. Such distribution might be caused by a slightly different buoyant density of MCPyV VLPs in comparison to Mouse Polyomaviral VLPs (CsCl concentration optimized for isolation of MPyV VLPs was used, where VLPs are present around the middle of the gradient). Refraction indexes of fractions were measured (Tab. 4.2). Fractions were combined based on refraction indexes and dot blot results into two aliquots, A (low concentration of VLPs) and B (high concentration of VLPs).

Aliquots were dialyzed overnight in B buffer and another sucrose cushion ultracentrifugation was applied (rotor SW41, 35,000 rpm, 3 hours) in order to purify and concentrate VLPs. After centrifugation, supernatant was removed and pellets were sonicated in 150 µl of B buffer.

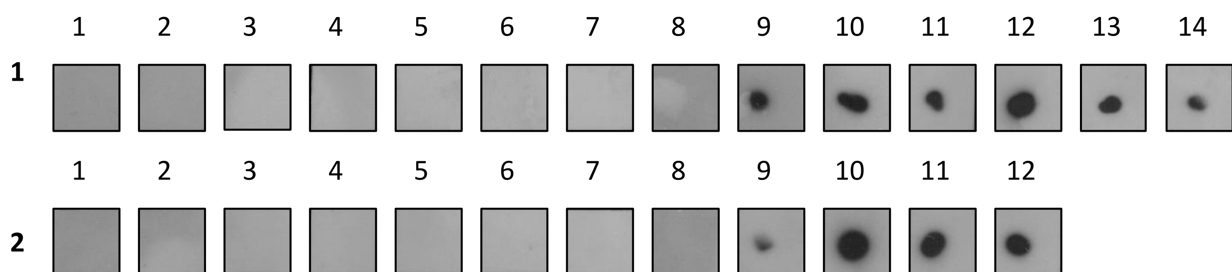


Fig.4.42: Dot blot of fractions of two CsCl gradients (1 and2) after isopycnic centrifugation. Antibody against VP1 of MCPyV was used as primary antibody. Goat antibody conjugated with HRP against mouse immunoglobulins was used as secondary antibody (dilution 1:1000). Film was exposed for 90 seconds. Fractions 1-9 from gradient 1 and 1-8 from gradient 2 were combined into aliquot A (low concentration). Fractions 10-14 from gradient 1 and 9-12 from gradient 2 were combined to aliquot B (high concentration).

Fractions of gradient 1	Refraction index	Aliquot	Fractions of gradient 2	Refraction index
1	1.368		1	1.377
2	1.377		2	1.376
3	1.374		3	1.371
4	1.371	A	4	1.369
5	1.369		5	1.366
6	1.365		6	1.366
7	1.363		7	1.364
8	1.364		8	1.363
9	1.363		9	1.361
10	1.362		10	1.360
11	1.361	B	11	1.357
12	1.359		12	1.356
13	1.358			
14	1.356			

Tab. 4.2: Refraction indexes of fractions from CsCl gradients and distribution of fractions into aliquots. MCPyV VLPs are found mainly in upper fractions of the CsCl gradient. In comparison to MPyV VLPs, the buoyant density is probably altered.

4.4.6.3. Yield characterisation

Several assays were applied to characterise VLPs aliquots. Protein concentration was measured by Bradford method, 0.14 µg/ml and 0.18 µg/ml found in aliquots A and B, respectively. Transmission electron microscopy (TEM) was applied to examine the morphology and stability of VLPs. Negative staining was used for visualisation, the whole process was performed by Mgr. Martin Fraiberk (Fig. 4.43).

Some VP1 proteins of polyomaviruses show the ability to bind erythrocytes and hemagglutination assay can be used to determine concentration of viral capsids. Capsids of MPyV bind to guinea pig erythrocytes, and the same was observed for MCPyV capsids (VP1 VLPs, Sauerová, diploma thesis, 2013). Hemagglutination assay was applied on VLPs aliquots (Fig. 4.44). Concentration of VLPs was determined using a formula (empirically determined for MPyV viral capsids):

$$HAU \cdot 200 \cdot 10^7 = particles/ml$$

Aliquot A did not prevent erythrocyte hemagglutination at all. Aliquot B prevented hemagglutination up to dilution 1:32 (corresponding to HAU 32), which corresponds to concentration $6.4 \cdot 10^{10}$ particles / ml. Since the formula was made for concentration determination of MPyV capsids, it should be considered as just an estimate in case of MCPyV VLPs.

Characterized aliquots were stored at -80°C.

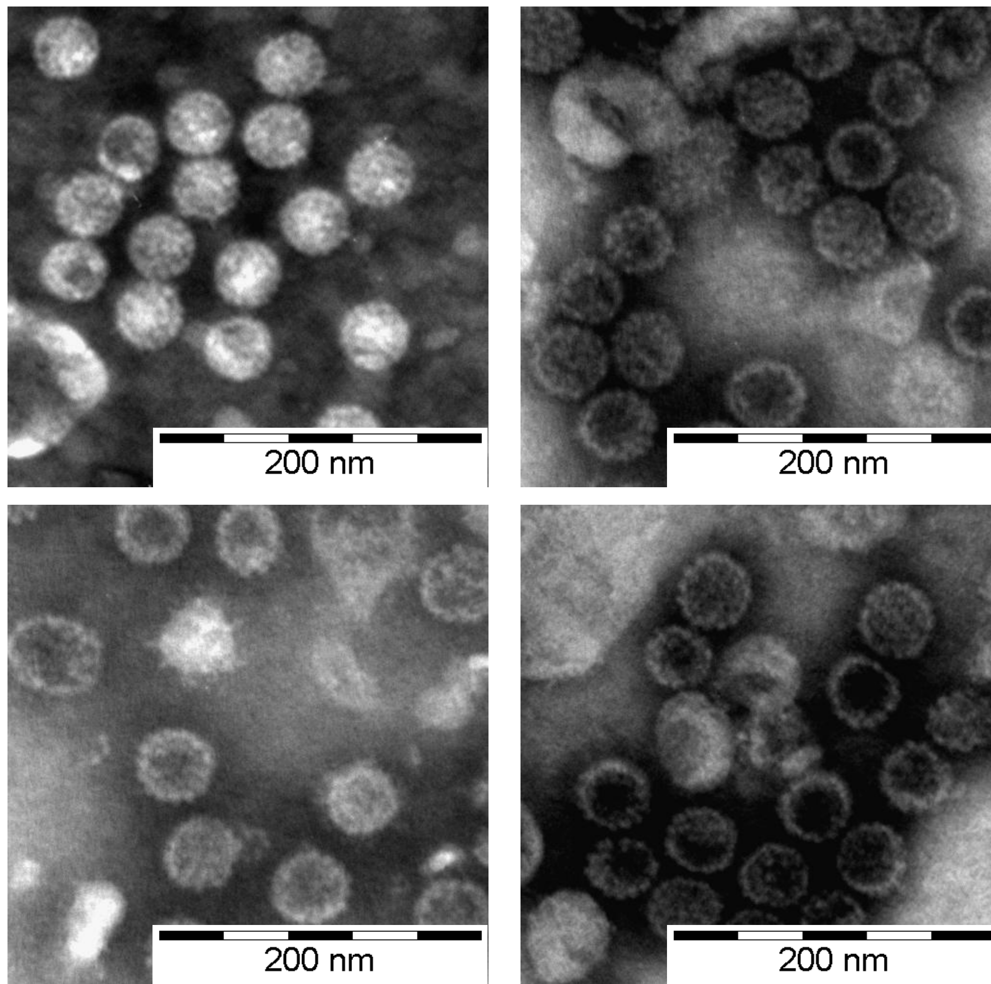


Fig.4.43: TEM of isolated VLPs from VLPs isolation. Samples were prepared and photographed by M. Fraiberk using negative staining. All pictures refer to aliquot B by (see text).

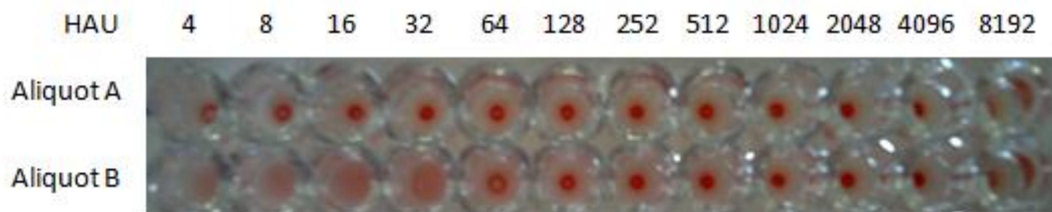


Fig.4.44: Hemagglutination assay of MCPyV VLPs. Aliquot A does not prevent hemaglutination of guinea pig erythrocytes. Aliquot B prevents hemaglutination of guinea pig erythrocytes up to dilution 1:32 (corresponding to HAU 32).

5. Discussion

5.1 Preparation of monoclonal antibody against N-terminal unique part of VP2 protein of BK virus

BK virus is an important human pathogen, pathogenicity of which affects growing number of people. Life cycle of this pathogen is, however, described only very poorly. Our laboratory conducts research on BKV life cycle. Concerning visualization of viral proteins, especially minor structural proteins, our current laboratory equipment is limited. Fusion proteins VP3 with EGFP were prepared by K. Podolská (diploma thesis, 2008). These constructs are useful in transfection assays for determination of cytotoxicity and other aspects of individual proteins. Visualization of minor structural proteins during infection is performed using a cross-reactive polyclonal serum against minor proteins of SV40. The specificity of this polyclonal antibody is low and so the need of monoclonal antibody specifically targeted to BKV minor proteins is evident.

Polyomaviral minor proteins VP2 and VP3 share majority of their sequence. Prepared monoclonal antibodies are targeted preferentially to the common region and so proteins VP2 and VP3 cannot be distinguished by staining with such antibody. Despite the efforts made many times to develop hybridoma line producing antibody directed against the unique part of VP2 protein, it never succeeded (H. Kasamatsu and J. Forstová, personal communication). The only successful attempt (Kasamatsu, 1982) ended in death of all hybridoma clones shortly after testing. Since this failure had happened repeatedly using unique parts of VP2 from various polyomaviruses, it seemed the main problem lied in low immunogenicity of this antigen. As described above, monoclonal antibody directed to this epitope of VP2 would be an invaluable tool to distinguish localization of VP2 and VP3 during infection. Ongoing attempts and new approaches are required.

5.1.1. Mouse immunization

Mice were immunized with unique part of VP2 protein fused with His-tag and S-tag expressed in bacteria (prepared by M. Verdánová, diploma thesis, 2011). S-tag is encoded in the pET29b plasmid (the used vector) as a part of the multiple cloning site. Removing of the sequence encoding S-tag would require additional and complicated cloning. S-tag fusion to the protein of interest was, however, not suspected to (and also did not) intefere with protein expression or purification since it is a small and commonly used affinity tag (Terpe, 2003). His-tag is localized at the C-terminus of the recombinant protein and it had two functions. First, it was used as a tag for protein affinity purification from bacterial lysates. So a relatively pure protein was used for mice immunization. That means mice were stimulated for production of antibodies directed to this one antigen only. Second, it probably served as an immunogenicity enhancer. An approach of antigen immunogenicity

enhancement by fusion with epitope that stimulates immune response is used in DNA vaccines (Tymciu et al., 2004), where problems with low immunogenicity are frequent. Successful use of this approach was also reported for standard immunization method (injection of protein solution) (Kumar et al., 1992). His-tag is usually considered as low-immunogenic or at least less immunogenic than the antigen of interest. On the other hand, repetitive character of His-tag rather implies higher immunogenicity and a case of enhancement of mouse immune response solely due to His-tag fusion was also reported (Khan et al., 2012).

Fortunately, immunogenicity enhancement was probably the case in this attempt as well. Both mice responded well to immunization and after three doses of intraperitoneal injection (which is a standard procedure) they were ready to be sacrificed for hybridoma preparation. The mouse with better response was boosted and hybridoma cells were prepared three days afterwards. Also, when the first generation of hybridomas was tested (hybridoma colonies produced directly from fusion), it revealed more than 130 clones positive for production of desired antibody (out of approximately 400 tested). In summary, two mice were successfully immunized. From one of them, hybridoma cell line was prepared with more than one hundred clones with positive signal for production of specific antibody.

5.1.2. Hybridoma maintenance

Hybridoma cells were successfully cultured and cloned up to third generation (three rounds of cloning using limited dilution). Often some clones died out entirely and the frequency of this was increasing together with increasing antibody specificity. Several times the whole generation of clones died and new one was prepared from frozen aliquots. This situation happened repeatedly and so attempts to prepare a hybridoma line were abandoned.

Preparation of polyclonal antibody could be a way for future attempts. The antigen ready for immunization was prepared in sufficient amount and purity. Immunogenicity of the antigen proved to be sufficient, so it could be used for generation of polyclonal serum in mouse or rabbit.

5.2 Preparation of monoclonal antibody against VP2 protein of Merkel Cell Polyomavirus

Merkel Cell Polyomavirus is a newly discovered polyomavirus and little is known about its life cycle, despite the fact that it is an object of a great scientific interest. Since no permissive cell line for production of the virus has been found till today, research is conducted mainly on the clinical basis. The need for data which could help us understand viral life cycle is enormous, not only it terms of

potential development of therapeutics but also in terms of finding ways to produce virus and study its infection at laboratory conditions. Our laboratory tends to participate in addressing these challenging questions. One of the ways is to study viral proteins and their impact on cells *in vitro*. For such research it is necessary to visualize these proteins.

In terms of structural proteins visualization, a monoclonal antibody against major protein VP1 (structural epitope) as well as a polyclonal antibody against protein VP1 (sequence epitope) were prepared by P. Sauerová (diploma thesis, 2013). Considerable attention is paid to research of polyomaviral minor proteins in our laboratory. The presence of these proteins will condition productive infection and their roles during infection differ among various polyomaviruses. To study MCPyV minor proteins, expression constructs fused with EGFP (J. Cibulka, diploma thesis, 2013) or FLAG (P. Sauerová, diploma thesis, 2013) were prepared. These constructs are used in transfection assays and minor proteins can be visualized using antibodies targeted to the fused tags. Although these constructs are successfully used in our laboratory, this approach is apparently not flawless. Fused tags combined with the tranfection process can affect expression, folding and localization of the minor proteins as well as their impact on cell culture. A monoclonal antibody targeted against native (and denatured) VP2 protein only is needed.

5.2.1. Mouse immunization

Two mice were immunized repeatedly by DNA vaccination (gene gun delivery of plasmid encoding DNA for VP2 protein of MCPyV). This method was chosen due to our bad experience with expression and purification of polyomaviral VP2 proteins (K. Podolská, diploma thesis, 2006, chapter 5.3 and others). Mice antibody response after this immunization was tested and was rather poor (data not shown). Subsequent immunization with peritoneal injection of protein solution (lysates of 3T3 cells transfected with the plasmid described above) was performed. Combining of DNA vaccination with standard intraperitoneal injection of antigen is an efficient way to enhance antibody response and is used widely (Lu et al., 2008). Combination of the two approaches resulted in positive immune response and generation of antibodies (as tested by immuno-dot blot with mouse blood sample). Mouse with stronger response was boosted with the same protein solution and sacrificed for hybridoma preparation three days afterwards.

5.2.2. Hybridoma maintenance

Hybridoma cells were tested by FACS analysis using Sf9 cells infected with recombinant baculovirus (producing the proteins of interest). Clones were tested on comparative basis (with negative and positive controls) in each tested plate (due to many changing sample parameters like number of

cells, efficiency of baculoviral infection, efficiency of staining, cells condition etc.). Positive clones were stored in liquid nitrogen. The most positive ones were cloned using limited dilution (one well to 96 wells on a feeder layer).

During the preparation process, bacterial contamination appeared in the culture. Since the contamination reappeared after thawing of other clones, it was probably caused by the contaminant presence in some equipment of common use when handling cell cultures (flow cabinet, cell incubator, pipettes etc.) or the contamination had happened early in the preparation process and was controlled over some time by the antibiotics mix added to HAT medium. Myeloma cells used for fusion (SP2/0 cell line) possess resistance against geneticin, this resistance is transmitted to hybridomas and so geneticin can be used to fight bacterial contamination. From then on, hybridomas were cultivated in HAT medium supplemented with 500 ng/µl of geneticin and bacterial infection was gradually eliminated. Some of the clones succumbed to the presence of geneticin (their resistance might get lost during long-term cultivation with no selection), but the rest prospered well. Antibody production seemed not to be affected by the presence of geneticin as measured by FACS analysis (after several days of treatment).

5.2.3. Collecting of antibody

After four rounds of cloning, some FACS samples showed regular staining (Fig. 4.9), which implied the antibody production was balanced and the production clone established. Nine such clones with strong signal were chosen. These clones were cultivated in larger scale (passaged gradually to a 25 cm^2 cultivation flask) and tested by various methods.

Culture medium from the clones was collected during passages or when replaced with fresh one. The medium was centrifuged in a refrigerated centrifuge to get rid of residual hybridoma cells or potential bacterial cells and supplemented with 0.05% sodium azide. Collected media from the nine clones were stored separately at 4°C. Media (presumably containing desired monoclonal antibodies) were tested for staining of various antigens (as primary antibody, see chapter 4.2.5.). Unfortunately, staining efficiency of all the media was very low even when incubation time in primary antibody was extended to overnight. This extension resulted in lower specificity (which could be observed in controls as well).

The antibody testing failure might be caused by several reasons. First, antibodies were not sufficiently concentrated for the laboratory use. Second, some of the clones tend to stain preferentially VP1 protein of MCPyV or other antigens used as negative controls. This altered specificity of antibody (targeting protein VP1 of MCPyV or other baculoviral or Sf9-cellular protein)

could occur since samples were tested for staining of complex antigens (Sf9 cells infected with a recombinant baculovirus producing VP1 and VP2 proteins of MCPyV) during the preparation process. This complex antigen was used for testing since expression of VP2 alone is not possible due to its cytotoxicity (Huerfano et al., 2010). Moreover, mouse immunization was performed using DNA vaccine and ph2m-transfected 3T3 cell lysates. In these systems target VP2 epitopes for antibodies might be different from epitopes in VP2 expressed in baculoviral system (used for hybridoma testing), mainly in accessibility of individual epitopes. More detailed analysis of preferential antibody epitope is needed.

Low concentration of the collected antibodies was probably a result of cultivation in larger scale. Hybridoma cells tend to lose their ability to produce antibody during passages (Howard and Bethell, 2009) since HAT medium does not select productive clones (selection is based only on complementation of metabolism deficit of myelomas by B lymphocytes). This is in agreement with decreased signal measured by FACS analysis for staining with antibody collected from clones cultivated in larger scale in comparison with the corresponding samples initially marked as established (data not shown).

Both problems with production level and stability described above can be solved by one more hybridoma cloning. One more round of separation and purification of individual productive clones should give rise to a clone sufficient in antibody production and stability. In case that most of the clones currently cultivated (in 25 cm^2 flasks) have already lost their ability to produce antibody, cloning could be performed from frozen aliquots stored shortly after initial FACS testing, in which antibody production should be conserved.

The presence of geneticin in culture medium (as described above) should not be underestimated. Even though no eminent influence on antibody production was observed after several days of treatment, it could have some impact on antigen binding as well. Lowering concentration of geneticin (or avoiding it completely) during cultivation might increase production level and staining efficiency.

5.3 Expression of recombinant truncated VP2 protein of BK virus in bacterial system

First step of monoclonal antibody preparation is a preparation and purification of antigen against which the antibody is supposed to be targeted. Expression and purification of BKV minor protein VP2 from bacteria was unsuccessfully performed earlier in our laboratory (K. Podolská, diploma thesis, 2008). Based on this experience and based on prediction of transmembrane domains in protein VP2

(Fig. 4.11A), a truncation of VP2 protein was suggested and performed. C-terminus of the protein was truncated, comprising 62 amino acids which include the most hydrophobic region of the three regions determined as transmembrane domains. A different C-terminal truncation – elimination of two out of three hydrophobic regions (resulting in the unique part only) of VP2 protein of BKV was performed and protein was successfully expressed and purified from bacteria by M. Verdánová (diploma thesis, 2011).

5.3.1. Cloning

Plasmid pET29b was used for cloning of truncated VP2 protein (pET29b was successfully used earlier by M. Verdánová for expression of VP2 unique). The plasmid encodes His-tag in its sequence, which is fused with the recombinant protein on its C-terminus and also S-tag fused on N-terminus. S-tag was not fused to the protein on some purpose, but it is encoded in the pET29b plasmid in its multiple cloning site. Sequence encoding S-tag can be removed by restriction cleavage when NheI restriction endonuclease is used for cloning. Restriction site for NheI is also present inside the sequence of DNA encoding tVP2 and so could not be used for cloning of the insert. S-tag fusion to the protein of interest was, however, not suspected to intefere with protein expression or purification since it is a small and commonly used affinity tag (Terpe, 2003). Later it even turned to be advantageous because it could be used in detection of N-terminal part of the expressed protein (see below).

5.3.2. Protein expression

Antibody against BK viral minor proteins is not available in our laboratory, neither commercially. A polyclonal serum targeted against minor proteins of SV40 cross-reacts with minor proteins of BKV (antibody cross-reactivity between antigens of these two viruses is a known fact, (Viscidi et al., 2003)), however, in case of BL-21 bacterial lysates it proved to be unspecific (data not shown) and could not be used. Thanks to fusion of the protein with C-terminal His-tag, antibody against His-tag could be used for protein detection of native as well as denatured proteins.

Protein expression under initial conditions (28°C, 0.1mM IPTG, 3 hours) produced very low amount of protein. Two plasmids originating from two colonies (11 and 12) of XL1 blue bacteria were tested with low protein yields in all experiments. Initial low yield of protein is not rare in protein expression, conditions optimization is often necessary. Within optimization process (using plasmid originating from colony 12), alternative temperatures for expression, alternative time of expression and IPTG concentration were being adjusted which resulted in equally low or even lower protein yield. An alternative protocol in which bacterial expression is induced at OD_{600} = 0.1 was performed as well, with equal results.

The His-tag is localized on C-terminus of the recombinant protein, which means it is transcribed and translated last. Transcription or translation failure could occur or the recombinant protein could be partially degraded in bacteria (which happens sometimes in recombinant protein expression). In any of these cases, His-tag would not be properly expressed. To determine whether some shorter products could be visualized in lysates, detection of S-tag was performed. S-tag is fused at N-terminus of the recombinant protein (due to setting of pET29b plasmid). Detection of S-tag in Western-blotted lysates revealed that the recombinant protein is expressed, although in low amount. Based on the S-tag detection, a part of the protein yield is degraded (degradative products molecular weight is mainly around 21 kDa and 19 kDa (Fig. 4.26) or smaller, around 15 kDa, (Fig. 4.24)) but the band with the strongest signal corresponds to the full length of the protein of interest (truncated VP2 fused with His-tag and S-tag, 29.6 kDa). This could mean that the problem with protein detection based on His-tag was caused either by low staining efficiency of primary antibody targeted against His-tag, by improper expression of His-tag or by partial degradation of the protein.

The recombinant protein could not be detected on SDS PAGE (Fig. 4.23) since no extra band was present in lysates of bacteria induced to expression in comparison to lysates of control bacteria (lacking the recombinant plasmid). Only Western blot immunodetection (in which background of other proteins signal is supressed, unlike in SDS PAGE) detected the expressed protein and only in cell lysates of sample bacteria. These results imply that the expression level of the recombinant protein must be very low. Staining of SDS PAGE gel after performance of Western blot with CBBG solution revealed that one band (corresponding to the molecular weight of the recombinant protein) was blotted with low efficiency. We were not able to distinguish whether lack of immunodetection signal on Western blot in case of native lysate is caused by protein absence or by blotting failure. To address this problem, purification using HIS-Select® Nickel Affinity Gel was performed. Native lysates were applied on the HIS-Select® Nickel Affinity Gel (cell lysates cannot be used for this method). No signal corresponding to the recombinant protein was detected in any step of the purification (only in the input sample).

VP2 protein of BK virus is a hydrophobic protein and has affinity to membranes. As described above, expression and purification of the whole sequence of VP2 failed (K. Podolská, diploma thesis, 2008). On the other hand, when two out of three hydrophobic regions were excluded, no problems occurred (M. Verdánová, diploma thesis, 2011). Truncation of the VP2 protein excluding one out of three hydrophobic domains is apparently not sufficient to push this protein into soluble fraction (native lysate) and it can only be found in the total protein (cell lysate). Moreover, the presence of His-tag in the amino acid sequence should not be underestimated. His-tag is frequently used in recombinant protein expression thanks to its easy purification on His-affinity gels or columns

(containing metal ions). His-tag only contains 6 amino acids and its impact on protein structure or other aspects is usually insignificant (Carson et al., 2007). But a comparative study of proteins expressed with no His-tag, C-terminal His-tag and N-terminal His-tag revealed that His-tag has negative impact on protein solubility, especially when localized on C-terminus (Woestenenk et al., 2004). The impact is not huge, but in case of protein with affinity to membranes like VP2, this could play an important role. The His-tag negative effect could be, however, reversed by fusion with additional tag which increases protein solubility (Hammarström et al., 2002).

The expression of truncated VP2 in bacteria did not prove suitable. A different expression system together with fusion with additional or entirely different tag (to improve protein solubility) could solve the problems and result in successful protein expression and purification.

5.4 Preparation of recombinant baculovirus for production of VP1 and VP2 proteins of Merkel Cell Polyomavirus

Preparation of virus-like particles (VLPs) was used as a method for easy protein production in this thesis. The prepared particles could of course be used in various studies of MCPyV capsids as well. Polyomaviral VLPs are artificial viral capsids prepared by simple expression of major structural protein VP1 (Salunke et al., 1986). This protein spontaneously forms pentamers and these subsequently form viral particles in the expression system. When one or both minor proteins (VP2 and VP3) are coexpressed with VP1, they also incorporate into these particles (Forstová, 1993).

Recombinant baculoviral expression system was used for preparation of VLPs consisting of proteins VP1 and VP2 of MCPyV. Preparation of VLPs in baculoviral expression system as a method for VP2 protein preparation was chosen due to several aspects. First, baculoviral system produces high yields of proteins and polyomaviral VLPs assemble successfully in this system (Forstová, 1993). Second, recombinant proteins can be detected in cell lysates, in intact Sf9 cells (used in FACS analysis) or VLPs can be easily purified by methods used for virus purification (using ultracentrifugation). Third, minor proteins are cytotoxic and hydrophobic when expressed individually (Huerfano et al., 2010). After incorporation into VLPs cytotoxicity is diminished as well as affinity to membranes and other hydrophobic structures.

Since MCPyV host cells have not been determined till today, our knowledge concerning genome sequence is deduced mainly from viral genome fragments incorporated into MCC carcinoma cells. These sequences cannot be efficiently expressed in expression systems and so codon modification was performed (in order to increase expression efficiency) in plasmids pwm (encoding VP1 protein of MCPyV) and ph2m (encoding VP2 protein of MCPyV) (Pastrana et al., 2009). These codon-modified

plasmids were used for generation of recombinant baculovirus MCPyV VP1/2. However, production of recombinant proteins VP1 and VP2 of MCPyV in Sf9 cells was somewhat lower than is usually seen in recombinant baculoviruses producing proteins of MPyV in our laboratory. Based on immunofluorescence results, practically every cell was infected when MOI 7 was used in baculoviral infection and so the lower production level could be caused by lower expression efficiency in Sf9 cells. Analogically, production of recombinant proteins of BK virus in baculoviral system (recombinant baculovirus BKV VP1/2 prepared by Hrušková and Stančíková, unpublished data) results in equal yields as for the baculovirus MCPyV VP1/2 (Fig. 4.37).

5.4.1. Production of VLPs

VLPs were purified using repeated ultracentrifugations (sucrose cushion and CsCl gradient). While this was the first time when VLPs consisting of VP1 and VP2 proteins of MCPyV were isolated in our laboratory, a protocol used for isolation of other polyomaviral VLPs was applied. This protocol had been successfully used in our laboratory for purification of various polymaviral virions or VLPs (MPyV, BKV, JCV, SV40). This is possible thanks to high similarity in morphology and size of various polyomaviral capsids (Sapp and Day, 2009). CsCl gradient used for the purification was, according to the protocol mentioned above, set to the refraction index of 1.365. After isopycnic ultracentrifugation, fractions of CsCl gradient were tested for VP1 protein presence by dot blot. This assay revealed presence of VLPs preferably in the upper half of the gradient. Refraction indexes were measured for each fraction. This measurement revealed that refraction index is close but not quite the same for MPyV and MCPyV VLPs. Produced VLPs were stable and their morphology wass correct as proven by electron microscopy observations. On the other hand, concentration of particles was low as was shown by Bradford method. Optimization of CsCl gradient ultracentrifugation could improve the yields and distribution of particles in the gradient.

Also, capability of VLPs to prevent hemagglutination of guinea pig erythrocytes was observed (which is in agreement with observation of P. Sauerová in VP1 only VLPs). This capability is thus shared by VP1 protein of MPyV and MCPyV, which is in agreement with proposed kinship between these two viruses (Feltkamp et al., 2007) as well as binding of sialyated glycans as coreceptors by MCPyV (Schowalter et al., 2011). This observation implies that hemagglutination assay can be used for determining concentration of capsids or particles of MCPyV. To determine exact concentration of viral particles, a formula has to be prepared for the actual experimental setting. In our laboratory, a formula prepared for determination of MPyV viral particles or VLPs is commonly used (part of a protocol made by Türler and Beard, 1985). This formula was used in this diploma thesis as well, so the calculated concentration is approximate and should be considered as such. Based on comparison

with results of Bradford assay and electron microscopy, the MPyV formula seems to be slightly underestimating. However, no conclusion can be made based on this single experiment. After MCPyV-specific formula is generated, hemagglutination assay will be a handy method for easy and fast determination of particles concentration.

6. Summary

1. Preparation of monoclonal antibody against N-terminal unique part of VP2 protein of BK virus

- Two mice were successfully immunized with antigen fused with His-tag
- Hybridoma cells were prepared by fusion of mouse splenocytes with myelomas
- Antibody production screening revealed over 130 clones positive for antibody of desired specificity
- Stable production clone was never established since hybridoma cells died out after three rounds of cloning

2. Preparation of monoclonal antibody against VP2 protein of Merkel Cell Polyomavirus

- Two mice were successfully immunized with DNA vaccine and cell lysates with expressed antigen
- Hybridoma cells were prepared by fusion of mouse splenocytes with myelomas
- Antibody production screening revealed clones positive for antibody of desired specificity
- Stable antibody production was observed in some clones after fourth cloning
- Nine clones were cultivated in larger scale and tested in order to specify and produce the antibody
- Antibody concentration and specificity was insufficient probably as a consequence of production loss during passages of hybridoma cells

3. Preparation of bacterial system for expression of C-terminally truncated VP2 protein of BK virus

- A vector suitable for expression of antigen fused with His-tag was prepared and verified
- The vector was successfully delivered (electroporated) into BL-21 DE3 bacteria suitable for induced expression
- Expression optimization was performed which resulted in slightly bigger yield of protein when IPTG concentration was augmented
- Purification of protein using HIS-Select® Nickel Affinity Gel was performed, results of which confirmed concerns about poor concentration of expressed protein

4. Preparation of recombinant baculoviral system for expression of VP1 and VP2 proteins of Merkel Cell Polyomavirus

- A plasmid carrying DNA encoding the two antigens under polyhedrin and p10 promoters was prepared and verified
- A bacmid (baculoviral genome) carrying DNA encoding the two antigens under polyhedrin and p10 promoters was prepared and verified

- Sf9 cells were electroporated with the recombinant bacmid which resulted in generation of recombinant baculovirus
- Viral stock was purified by plaque assay and multiple infections were performed in order to augment viral concentration
- Production of recombinant proteins was verified using various methods of protein detection
- Virus-like particles assembled in infected Sf9 cells were isolated using ultracentrifugation
- Purified particles stock was characterized using various methods
- Particles morphology was verified by electron microscopy

7. References

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8. Supplement

Sequencing data:

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11              ATGGGTGCTGCTCTAGCACTTTTGGGGGACCTAGTTGCCAGTGTATCTGAGGCTGCTGCT 60
tVP2            ATGGGTGCTGCTCTAGCACTTTTGGGGGACCTAGTTGCCAGTGTATCTGAGGCTGCTGCT 60
12              ATGGGTGCTGCTCTAGCACTTTTGGGGGACCTAGTTGCCAGTGTATCTGAGGCTGCTGCT 60
                ************************************************************

11              GCCACAGGATTTTCAGTGGCTGAAATTGCTGCTGGGGAGGCTGCTGCTGCTATAGAAGTT 120
tVP2            GCCACAGGATTTTCAGTGGCTGAAATTGCTGCTGGGGAGGCTGCTGCTGCTATAGAAGTT 120
12              GCCACAGGATTTTCAGTGGCTGAAATTGCTGCTGGGGAGGCTGCTGCTGCTATAGAAGTT 120
                ************************************************************

11              CAAATTGCATCCCTTGCTACTGTAGAGGGCATAACAAGTACCTCAGAGGCTATAGCTGCT 180
tVP2            CAAATTGCATCCCTTGCTACTGTAGAGGGCATAACAAGTACCTCAGAGGCTATAGCTGCT 180
12              CAAATTGCATCCCTTGCTACTGTAGAGGGCATAACAAGTACCTCAGAGGCTATAGCTGCT 180
                ************************************************************

11              ATAGGCCTAACTCCTCAAACATATGCTGTAATTGCTGGTGCTCCTGGGGCTATTGCTGGG 240
tVP2            ATAGGCCTAACTCCTCAAACATATGCTGTAATTGCTGGTGCTCCTGGGGCTATTGCTGGG 240
12              ATAGGCCTAACTCCTCAAACATATGCTGTAATTGCTGGTGCTCCTGGGGCTATTGCTGGG 240
                ************************************************************

11              TTTGCTGCTTTAATTCAAACTGTTAGTGGTATTAGTTCCTTGGCTCAAGTAGGGTATAGG 300
tVP2            TTTGCTGCTTTAATTCAAACTGTTAGTGGTATTAGTTCCTTGGCTCAAGTAGGGTATAGG 300
12              TTTGCTGCTTTAATTCAAACTGTTAGTGGTATTAGTTCCTTGGCTCAAGTAGGGTATAGG 300
                ************************************************************

11              TTCTTTAGTGATTGGGATCACAAAGTTTCCACTGTAGGCCTCTATCAGCAATCAGGCATG 360
tVP2            TTCTTTAGTGATTGGGATCACAAAGTTTCCACTGTAGGCCTCTATCAGCAATCAGGCATG 360
12              TTCTTTAGTGATTGGGATCACAAAGTTTCCACTGTAGGCCTCTATCAGCAATCAGGCATG 360
                ************************************************************

11              GCTTTGGAATTGTTTAACCCAGATGAGTACTATGATATTCTGTTTCCTGGTGTAAATACT 420
tVP2            GCTTTGGAATTGTTTAACCCAGATGAGTACTATGATATTCTGTTTCCTGGTGTAAATACT 420
12              GCTTTGGAATTGTTTAACCCAGATGAGTACTATGATATTCTGTTTCCTGGTGTAAATACT 420
                ************************************************************

11              TTTGTTAATAATATTCAATACCTTGATCCTAGGCATTGGGGTCCTTCTTTGTTTGCTACT 480
tVP2            TTTGTTAATAATATTCAATACCTTGATCCTAGGCATTGGGGTCCTTCTTTGTTTGCTACT 480
12              TTTGTTAATAATATTCAATACCTTGATCCTAGGCATTGGGGTCCTTCTTTGTTTGCTACT 480
                ************************************************************

11              ATTTCCCAGGCTTTGTGGCATGTTATTAGGGATGATATACCTTCTATAACCTCACAGGAA 540
tVP2            ATTTCCCAGGCTTTGTGGCATGTTATTAGGGATGATATACCTTCTATAACCTCACAGGAA 540
12              ATTTCCCAGGCTTTGTGGCATGTTATTAGGGATGATATACCTTCTATAACCTCACAGGAA 540
                ************************************************************

11              TTGCAGAGAAGAACAGAAAGATTTTTTAGAGACTCCTTGGCTAGATTTTTGGAGGAAACT 600
tVP2            TTGCAGAGAAGAACAGAAAGATTTTTTAGAGACTCCTTGGCTAGATTTTTGGAGGAAACT 600
12              TTGCAGAGAAGAACAGAAAGATTTTTTAGAGACTCCTTGGCTAGATTTTTGGAGGAAACT 600
                ************************************************************

11              ACCTGGACAATTGTAAATGCCCCTATAAACTTTTATAATTATATTCAACAATATTATTCT 660
tVP2            ACCTGGACAATTGTAAATGCCCCTATAAACTTTTATAATTATATTCAACAATATTATTCT 660
12              ACCTGGACAATTGTAAATGCCCCTATAAACTTTTATAATTATATTCAACAATATTATTCT 660
                ************************************************************

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tVP2            GATCTTTCCCCTATTAGGCCCTCAATGGTTAGACAAGTAGCTGAAAGGGAAGGTACCCGT 720
12              GATCTTTCCCCTATTAGGCCCTCAATGGTTAGACAAGTAGCTGAAAGGGAAGGTACCCGT 720
                ************************************************************

11              GTACATTTTGGCCATACTTATAGTATAGATGATGCTGACAGTATAGAAGAAGTTACACAA 780
tVP2            GTACATTTTGGCCATACTTATAGTATAGATGATGCTGACAGTATAGAAGAAGTTACACAA 780
12              GTACATTTTGGCCATACTTATAGTATAGATGATGCTGACAGTATAGAAGAAGTTACACAA 780
                ************************************************************

11              AGAATGGACTTAAGAAATCAACAAAGTGTACATTCAGGAGAGTTTATAGAAAAAACTATT 840
tVP2            AGAATGGACTTAAGAAATCAACAAAGTGTACATTCAGGAGAGTTTATAGAAAAAACTATT 840
12              AGAATGGACTTAAGAAATCAACAAAGTGTACATTCAGGAGAGTTTATAGAAAAAACTATT 840
                ************************************************************

11              GCCCCAGGAGGTGCTAATCAAAGAACT 867
tVP2            GCCCCAGGAGGTGCTAATCAAAGAACT 867
12              GCCCCAGGAGGTGCTAATCAAAGAACT 867
                ***************************
```

Fig. S1: Sequencing results for inserted tVP2 DNA. The inserted sequences from bacterial colonies no.11, 12 were identical to the template sequence (tVP2). Alignment was performed in ClusthalW2.

```
11          ATGGCCCCGAAGCGCAAGGCCAGCAGTACATGCAAGACCCCAAAGCGCCAGTGCATCCCG 60
VP1         ATGGCCCCGAAGCGCAAGGCCAGCAGTACATGCAAGACCCCAAAGCGCCAGTGCATCCCG 60
            ************************************************************

11          AAACCCGGCTGTTGTCCCAACGTCGCAAGCGTCCCGAAGCTGTTGGTCAAGGGCGGCGTC 120
VP1         AAACCCGGCTGTTGTCCCAACGTCGCAAGCGTCCCGAAGCTGTTGGTCAAGGGCGGCGTC 120
            ************************************************************

11          GAGGTGCTGAGCGTCGTCACCGGCGAGGACTCAATCACACAAATCGAACTCTACCTCAAC 180
VP1         GAGGTGCTGAGCGTCGTCACCGGCGAGGACTCAATCACACAAATCGAACTCTACCTCAAC 180
            ************************************************************

11          CCACGCATGGGCGTCAACAGTCCCGACTTGCCCACCACCAGCAATTGGTACACCTACACC 240
VP1         CCACGCATGGGCGTCAACAGTCCCGACTTGCCCACCACCAGCAATTGGTACACCTACACC 240
            ************************************************************

11          TACGATCTGCAACCCAAAGGCAGCAGCCCGGACCAGCCAATCAAAGAGAACCTGCCCGCC 300
VP1         TACGATCTGCAACCCAAAGGCAGCAGCCCGGACCAGCCAATCAAAGAGAACCTGCCCGCC 300
            ************************************************************

11          TATTCCGTCGCCCGCGTCAGCCTGCCCATGTTGAACGAAGACATCACATGCGATACCCTG 360
VP1         TATTCCGTCGCCCGCGTCAGCCTGCCCATGTTGAACGAAGACATCACATGCGATACCCTG 360
            ************************************************************

11          CAAATGTGGGAAGCCATCAGCGTCAAGACCGAGGTGGTCGGCATCAGCAGCCTGATCAAC 420
VP1         CAAATGTGGGAAGCCATCAGCGTCAAGACCGAGGTGGTCGGCATCAGCAGCCTGATCAAC 420
            ************************************************************

11          GTCCACTACTGGGATATGAAGCGCGTCCACGACTACGGCGCCGGCATCCCGGTCAGCGGC 480
VP1         GTCCACTACTGGGATATGAAGCGCGTCCACGACTACGGCGCCGGCATCCCGGTCAGCGGC 480
            ************************************************************

11          GTCAACTATCACATGTTCGCAATCGGCGGCGAGCCGTTGGACCTGCAAGGGCTGGTCCTG 540
VP1         GTCAACTATCACATGTTCGCAATCGGCGGCGAGCCGTTGGACCTGCAAGGGCTGGTCCTG 540
            ************************************************************

11          GACTATCAGACCCAGTACCCCAAGACCACCAACGGCGGCCCCATCACCATCGAGACCGTG 600
VP1         GACTATCAGACCCAGTACCCCAAGACCACCAACGGCGGCCCCATCACCATCGAGACCGTG 600
            ************************************************************

11          CTCGGCCGCAAGATGACCCCGAAGAACCAGGGGTTGGACCCACAAGCCAAGGCCAAGCTG 660
VP1         CTCGGCCGCAAGATGACCCCGAAGAACCAGGGGTTGGACCCACAAGCCAAGGCCAAGCTG 660
            ************************************************************

11          GACAAGGACGGCAACTACCCCATCGAGGTCTGGTGCCCCGACCCGAGCAAGAACGAGAAT 720
VP1         GACAAGGACGGCAACTACCCCATCGAGGTCTGGTGCCCCGACCCGAGCAAGAACGAGAAT 720
            ************************************************************

11          TCCCGCTACTACGGCAGCATCCAAACCGGGAGCCAGACCCCGACCGTGTTGCAGTTCAGC 780
VP1         TCCCGCTACTACGGCAGCATCCAAACCGGGAGCCAGACCCCGACCGTGTTGCAGTTCAGC 780
            ************************************************************

11          AACACCCTGACCACCGTGCTGCTGGACGAAAACGGCGTCGGCCCACTGTGCAAGGGCGAT 840
VP1         AACACCCTGACCACCGTGCTGCTGGACGAAAACGGCGTCGGCCCACTGTGCAAGGGCGAT 840
            ************************************************************

11          GGCCTGTTCATCTCATGCGCCGATATCGTCGGATTCCTGTTCAAGACATCCGGCAAGATG 900
VP1         GGCCTGTTCATCTCATGCGCCGATATCGTCGGATTCCTGTTCAAGACATCCGGCAAGATG 900
            ************************************************************

11          GCCTTGCACGGCCTGCCACGCTACTTCAACGTCACCCTGCGAAAGCGCTGGGTCAAGAAT 960
VP1         GCCTTGCACGGCCTGCCACGCTACTTCAACGTCACCCTGCGAAAGCGCTGGGTCAAGAAT 960
            ************************************************************

11          CCATATCCCGTGGTCAACCTGATCAATAGCTTGTTCAGCAATCTGATGCCCAAGGTCAGC 1020
VP1         CCATATCCCGTGGTCAACCTGATCAATAGCTTGTTCAGCAATCTGATGCCCAAGGTCAGC 1020
            ************************************************************

11          GGCCAGCCCATGGAGGGCAAGGACAACCAAGTGGAGGAAGTCCGCATCTACGAAGGCAGC 1080
VP1         GGCCAGCCCATGGAGGGCAAGGACAACCAAGTGGAGGAAGTCCGCATCTACGAAGGCAGC 1080
            ************************************************************

11          GAGCAGCTGCCCGGCGACCCCGACATCGTGCGGTTCCTGGACAAGTTCGGCCAAGAAAAG 1140
VP1         GAGCAGCTGCCCGGCGACCCCGACATCGTGCGGTTCCTGGACAAGTTCGGCCAAGAAAAG 1140
            ************************************************************

11          ACCGTCTATCCCAAACCAAGCGTCGCACCCGCCGCCGTGACATTCCAGTCCAACCAGCAA 1200
VP1         ACCGTCTATCCCAAACCAAGCGTCGCACCCGCCGCCGTGACATTCCAGTCCAACCAGCAA 1200
            ************************************************************

11          GACAAAGGCAAGGCCCCGCTGAAGGGCCCGCAAAAGGCAAGCCAGAAGGAGTCACAGACG 1260
VP1         GACAAAGGCAAGGCCCCGCTGAAGGGCCCGCAAAAGGCAAGCCAGAAGGAGTCACAGACG 1260
            ************************************************************

11          CAGGAGCTATGA 1272
VP1         CAGGAGCTATGA 1272
            ************
```

Fig. S2: Sequencing results for insert VP1 to be used in insertion into pFastBac™. PCR method was used for DNA preparation (see chapter 4.4.1.). The inserted sequence (11) was identical to the template sequence (VP1). Two sets of primers were used to cover the whole sequence. Alignment was performed in ClusthalW2.

```
12              ATGGGCGGGATCATTACCCTGCTCGCAAACATCGGCGAGATCGCCACCGAGCTGTCCGCA 60
VP2             ATGGGCGGGATCATTACCCTGCTCGCAAACATCGGCGAGATCGCCACCGAGCTGTCCGCA 60
                ************************************************************

12              ACAACCGGCGTGACACTGGAAGCCATCTTGACCGGCGAGGCCCTGGCCGCACTCGAGGCC 120
VP2             ACAACCGGCGTGACACTGGAAGCCATCTTGACCGGCGAGGCCCTGGCCGCACTCGAGGCC 120
                ************************************************************

12              GAGATTAGTAGCCTGATGACCATCGAAGGCATCAGCGGGATCGAAGCCCTGGCACAGCTG 180
VP2             GAGATTAGTAGCCTGATGACCATCGAAGGCATCAGCGGGATCGAAGCCCTGGCACAGCTG 180
                ************************************************************

12              GGCTTTACCGCCGAGCAATTCAGCAACTTCAGCCTGGTCGCCAGCCTCGTCAATCAGGGC 240
VP2             GGCTTTACCGCCGAGCAATTCAGCAACTTCAGCCTGGTCGCCAGCCTCGTCAATCAGGGC 240
                ************************************************************

12              CTGACCTACGGGTTCATCTTGCAAACCGTGTCCGGCATCGGGAGCCTGATCACCGTCGGC 300
VP2             CTGACCTACGGGTTCATCTTGCAAACCGTGTCCGGCATCGGGAGCCTGATCACCGTCGGC 300
                ************************************************************

12              GTCCGCCTGAGCAGGGAACAGGTCAGCCTGGTCAACCGCGACGTCAGCTGGGTCGGCTCC 360
VP2             GTCCGCCTGAGCAGGGAACAGGTCAGCCTGGTCAACCGCGACGTCAGCTGGGTCGGCTCC 360
                ************************************************************

12              AACGAAGTCCTGCGCCACGCCTTGATGGCATTCTCACTGGACCCGCTCCAATGGGAGAAC 420
VP2             AACGAAGTCCTGCGCCACGCCTTGATGGCATTCTCACTGGACCCGCTCCAATGGGAGAAC 420
                ************************************************************

12              AGCCTGCTGCACAGCGTCGGCCAGGACATCTTCAACAGCCTGAGCCCCACAAGCCGCCTG 480
VP2             AGCCTGCTGCACAGCGTCGGCCAGGACATCTTCAACAGCCTGAGCCCCACAAGCCGCCTG 480
                ************************************************************

12              CAAATCCAGAGCAACTTGGTCAACCTCATCCTGAACTCAAGATGGGTGTTCCAAACCACC 540
VP2             CAAATCCAGAGCAACTTGGTCAACCTCATCCTGAACTCAAGATGGGTGTTCCAAACCACC 540
                ************************************************************

12              GCCAGCCAGAACCAGGGGCTGCTGAGCGGCGAAGCCATCCTGATCCCCGAACACATCGGC 600
VP2             GCCAGCCAGAACCAGGGGCTGCTGAGCGGCGAAGCCATCCTGATCCCCGAACACATCGGC 600
                ************************************************************

12              GGCACCCTGCAACAGCAGACACCCGACTGGCTGCTGCCACTGGTGCTGGGGTTGTCCGGC 660
VP2             GGCACCCTGCAACAGCAGACACCCGACTGGCTGCTGCCACTGGTGCTGGGGTTGTCCGGC 660
                ************************************************************

12              TACATCAGCCCCGAGCTGCAAGTCATCGAGGACGGGACAAAGAAGAAGTCAATCATTCAT 720
VP2             TACATCAGCCCCGAGCTGCAAGTCATCGAGGACGGGACAAAGAAGAAGTCAATCATTCAT 720
                ************************************************************

12              CTCTGA 726
VP2             CTCTGA 726
                ******
```

Fig. S3: Sequencing results for insert VP2 to be used in insertion into pFastBac™. PCR method was used for DNA preparation (see chapter 4.4.1.). Inserted sequence was identical to template sequence. Alignment was performed in ClusthalW2.

A:

```
B               ATGGCCCCGAAGCGCAAGGCCAGCAGTACATGCAAGACCCCAAAGCGCCAGTGCATCCCG 60
VP1             ATGGCCCCGAAGCGCAAGGCCAGCAGTACATGCAAGACCCCAAAGCGCCAGTGCATCCCG 60
C               ATGGCCCCGAAGCGCAAGGCCAGCAGTACATGCAAGACCCCAAAGCGCCAGTGCATCCCG 60
                ************************************************************

B               AAACCCGGCTGTTGTCCCAACGTCGCAAGCGTCCCGAAGCTGTTGGTCAAGGGCGGCGTC 120
VP1             AAACCCGGCTGTTGTCCCAACGTCGCAAGCGTCCCGAAGCTGTTGGTCAAGGGCGGCGTC 120
C               AAACCCGGCTGTTGTCCCAACGTCGCAAGCGTCCCGAAGCTGTTGGTCAAGGGCGGCGTC 120
                ************************************************************

B               GAGGTGCTGAGCGTCGTCACCGGCGAGGACTCAATCACACAAATCGAACTCTACCTCAAC 180
VP1             GAGGTGCTGAGCGTCGTCACCGGCGAGGACTCAATCACACAAATCGAACTCTACCTCAAC 180
C               GAGGTGCTGAGCGTCGTCACCGGCGAGGACTCAATCACACAAATCGAACTCTACCTCAAC 180
                ************************************************************

B               CCACGCATGGGCGTCAACAGTCCCGACTTGCCCACCACCAGCAATTGGTACACCTACACC 240
VP1             CCACGCATGGGCGTCAACAGTCCCGACTTGCCCACCACCAGCAATTGGTACACCTACACC 240
C               CCACGCATGGGCGTCAACAGTCCCGACTTGCCCACCACCAGCAATTGGTACACCTACACC 240
                ************************************************************

B               TACGATCTGCAACCCAAAGGCAGCAGCCCGGACCAGCCAATCAAAGAGAACCTGCCCGCC 300
VP1             TACGATCTGCAACCCAAAGGCAGCAGCCCGGACCAGCCAATCAAAGAGAACCTGCCCGCC 300
C               TACGATCTGCAACCCAAAGGCAGCAGCCCGGACCAGCCAATCAAAGAGAACCTGCCCGCC 300
                ************************************************************

B               TATTCCGTCGCCCGCGTCAGCCTGCCCATGTTGAACGAAGACATCACATGCGATACCCTG 360
VP1             TATTCCGTCGCCCGCGTCAGCCTGCCCATGTTGAACGAAGACATCACATGCGATACCCTG 360
C               TATTCCGTCGCCCGCGTCAGCCTGCCCATGTTGAACGAAGACATCACATGCGATACCCTG 360
                ************************************************************

B               CAAATGTGGGAAGCCATCAGCGTCAAGACCGAGGTGGTCGGCATCAGCAGCCTGATCAAC 420
VP1             CAAATGTGGGAAGCCATCAGCGTCAAGACCGAGGTGGTCGGCATCAGCAGCCTGATCAAC 420
C               CAAATGTGGGAAGCCATCAGCGTCAAGACCGAGGTGGTCGGCATCAGCAGCCTGATCAAC 420
                ************************************************************

B               GTCCACTACTGGGATATGAAGCGCGTCCACGACTACGGCGCCGGCATCCCGGTCAGCGGC 480
VP1             GTCCACTACTGGGATATGAAGCGCGTCCACGACTACGGCGCCGGCATCCCGGTCAGCGGC 480
C               GTCCACTACTGGGATATGAAGCGCGTCCACGACTACGGCGCCGGCATCCCGGTCAGCGGC 480
                ************************************************************

B               GTCAACTATCACATGTTCGCAATCGGCGGCGAGCCGTTGGACCTGCAAGGGCTGGTCCTG 540
VP1             GTCAACTATCACATGTTCGCAATCGGCGGCGAGCCGTTGGACCTGCAAGGGCTGGTCCTG 540
C               GTCAACTATCACATGTTCGCAATCGGCGGCGAGCCGTTGGACCTGCAAGGGCTGGTCCTG 540
                ************************************************************

B               GACTATCAGACCCAGTACCCCAAGACCACCAACGGCGGCCCCATCACCATCGAGACCGTG 600
VP1             GACTATCAGACCCAGTACCCCAAGACCACCAACGGCGGCCCCATCACCATCGAGACCGTG 600
C               GACTATCAGACCCAGTACCCCAAGACCACCAACGGCGGCCCCATCACCATCGAGACCGTG 600
                ************************************************************

B               CTCGGCCGCAAGATGACCCCGAAGAACCAGGGGTTGGACCCACAAGCCAAGGCCAAGCTG 660
VP1             CTCGGCCGCAAGATGACCCCGAAGAACCAGGGGTTGGACCCACAAGCCAAGGCCAAGCTG 660
C               CTCGGCCGCAAGATGACCCCGAAGAACCAGGGGTTGGACCCACAAGCCAAGGCCAAGCTG 660
                ************************************************************

B               GACAAGGACGGCAACTACCCCATCGAGGTCTGGTGCCCCGACCCGAGCAAGAACGAGAAT 720
VP1             GACAAGGACGGCAACTACCCCATCGAGGTCTGGTGCCCCGACCCGAGCAAGAACGAGAAT 720
C               GACAAGGACGGCAACTACCCCATCGAGGTCTGGTGCCCCGACCCGAGCAAGAACGAGAAT 720
                ************************************************************

B               TCCCGCTACTACGGCAGCATCCAAACCGGGAGCCAGACCCCGACCGTGTTGCAGTTCAGC 780
VP1             TCCCGCTACTACGGCAGCATCCAAACCGGGAGCCAGACCCCGACCGTGTTGCAGTTCAGC 780
C               TCCCGCTACTACGGCAGCATCCAAACCGGGAGCCAGACCCCGACCGTGTTGCAGTTCAGC 780
                ************************************************************

B               AACACCCTGACCACCGTGCTGCTGGACGAAAACGGCGTCGGCCCACTGTGCAAGGGCGAT 840
VP1             AACACCCTGACCACCGTGCTGCTGGACGAAAACGGCGTCGGCCCACTGTGCAAGGGCGAT 840
C               AACACCCTGACCACCGTGCTGCTGGACGAAAACGGCGTCGGCCCACTGTGCAAGGGCGAT 840
                ************************************************************

B               GGCCTGTTCATCTCATGCGCCGATATCGTCGGATTCCTGTTCAAGACATCCGGCAAGATG 900
VP1             GGCCTGTTCATCTCATGCGCCGATATCGTCGGATTCCTGTTCAAGACATCCGGCAAGATG 900
C               GGCCTGTTCATCTCATGCGCCGATATCGTCGGATTCCTGTTCAAGACATCCGGCAAGATG 900
                ************************************************************

B               GCCTTGCACGGCCTGCCACGCTACTTCAACGTCACCCTGCGAAAGCGCTGGGTCAAGAAT 960
VP1             GCCTTGCACGGCCTGCCACGCTACTTCAACGTCACCCTGCGAAAGCGCTGGGTCAAGAAT 960
C               GCCTTGCACGGCCTGCCACGCTACTTCAACGTCACCCTGCGAAAGCGCTGGGTCAAGAAT 960
                ************************************************************

B               CCATATCCCGTGGTCAACCTGATCAATAGCTTGTTCAGCAATCTGATGCCCAAGGTCAGC 1020
VP1             CCATATCCCGTGGTCAACCTGATCAATAGCTTGTTCAGCAATCTGATGCCCAAGGTCAGC 1020
C               CCATATCCCGTGGTCAACCTGATCAATAGCTTGTTCAGCAATCTGATGCCCAAGGTCAGC 1020
                ************************************************************

B               GGCCAGCCCATGGAGGGCAAGGACAACCAAGTGGAGGAAGTCCGCATCTACGAAGGCAGC 1080
VP1             GGCCAGCCCATGGAGGGCAAGGACAACCAAGTGGAGGAAGTCCGCATCTACGAAGGCAGC 1080
C               GGCCAGCCCATGGAGGGCAAGGACAACCAAGTGGAGGAAGTCCGCATCTACGAAGGCAGC 1080
                ************************************************************

B               GAGCAGCTGCCCGGCGACCCCGACATCGTGCGGTTCCTGGACAAGTTCGGCCAAGAAAAG 1140
VP1             GAGCAGCTGCCCGGCGACCCCGACATCGTGCGGTTCCTGGACAAGTTCGGCCAAGAAAAG 1140
C               GAGCAGCTGCCCGGCGACCCCGACATCGTGCGGTTCCTGGACAAGTTCGGCCAAGAAAAG 1140
                ************************************************************

B               ACCGTCTATCCCAAACCAAGCGTCGCACCCGCCGCCGTGACATTCCAGTCCAACCAGCAA 1200
VP1             ACCGTCTATCCCAAACCAAGCGTCGCACCCGCCGCCGTGACATTCCAGTCCAACCAGCAA 1200
C               ACCGTCTATCCCAAACCAAGCGTCGCACCCGCCGCCGTGACATTCCAGTCCAACCAGCAA 1200
                ************************************************************

B               GACAAAGGCAAGGCCCCGCTGAAGGGCCCGCAAAAGGCAAGCCAGAAGGAGTCACAGACG 1260
VP1             GACAAAGGCAAGGCCCCGCTGAAGGGCCCGCAAAAGGCAAGCCAGAAGGAGTCACAGACG 1260
C               GACAAAGGCAAGGCCCCGCTGAAGGGCCCGCAAAAGGCAAGCCAGAAGGAGTCACAGACG 1260
                ************************************************************

B               CAGGAGCTATGA 1272
VP1             CAGGAGCTATGA 1272
C               CAGGAGCTATGA 1272
                ************
```

B:

```
B      ATGGGCGGGATCATTACCCTGCTCGCAAACATCGGCGAGATCGCCACCGAGCTGTCCGCA 60
VP2    ATGGGCGGGATCATTACCCTGCTCGCAAACATCGGCGAGATCGCCACCGAGCTGTCCGCA 60
C      ATGGGCGGGATCATTACCCTGCTCGCAAACATCGGCGAGATCGCCACCGAGCTGTCCGCA 60
       ************************************************************

B      ACAACCGGCGTGACACTGGAAGCCATCTTGACCGGCGAGGCCCTGGCCGCACTCGAGGCC 120
VP2    ACAACCGGCGTGACACTGGAAGCCATCTTGACCGGCGAGGCCCTGGCCGCACTCGAGGCC 120
C      ACAACCGGCGTGACACTGGAAGCCATCTTGACCGGCGAGGCCCTGGCCGCACTCGAGGCC 120
       ************************************************************

B      GAGATTAGTAGCCTGATGACCATCGAAGGCATCAGCGGGATCGAAGCCCTGGCACAGCTG 180
VP2    GAGATTAGTAGCCTGATGACCATCGAAGGCATCAGCGGGATCGAAGCCCTGGCACAGCTG 180
C      GAGATTAGTAGCCTGATGACCATCGAAGGCATCAGCGGGATCGAAGCCCTGGCACAGCTG 180
       ************************************************************

B      GGCTTTACCGCCGAGCAATTCAGCAACTTCAGCCTGGTCGCCAGCCTCGTCAATCAGGGC 240
VP2    GGCTTTACCGCCGAGCAATTCAGCAACTTCAGCCTGGTCGCCAGCCTCGTCAATCAGGGC 240
C      GGCTTTACCGCCGAGCAATTCAGCAACTTCAGCCTGGTCGCCAGCCTCGTCAATCAGGGC 240
       ************************************************************

B      CTGACCTACGGGTTCATCTTGCAAACCGTGTCCGGCATCGGGAGCCTGATCACCGTCGGC 300
VP2    CTGACCTACGGGTTCATCTTGCAAACCGTGTCCGGCATCGGGAGCCTGATCACCGTCGGC 300
C      CTGACCTACGGGTTCATCTTGCAAACCGTGTCCGGCATCGGGAGCCTGATCACCGTCGGC 300
       ************************************************************

B      GTCCGCCTGAGCAGGGAACAGGTCAGCCTGGTCAACCGCGACGTCAGCTGGGTCGGCTCC 360
VP2    GTCCGCCTGAGCAGGGAACAGGTCAGCCTGGTCAACCGCGACGTCAGCTGGGTCGGCTCC 360
C      GTCCGCCTGAGCAGGGAACAGGTCAGCCTGGTCAACCGCGACGTCAGCTGGGTCGGCTCC 360
       ************************************************************

B      AACGAAGTCCTGCGCCACGCCTTGATGGCATTCTCACTGGACCCGCTCCAATGGGAGAAC 420
VP2    AACGAAGTCCTGCGCCACGCCTTGATGGCATTCTCACTGGACCCGCTCCAATGGGAGAAC 420
C      AACGAAGTCCTGCGCCACGCCTTGATGGCATTCTCACTGGACCCGCTCCAATGGGAGAAC 420
       ************************************************************

B      AGCCTGCTGCACAGCGTCGGCCAGGACATCTTCAACAGCCTGAGCCCCACAAGCCGCCTG 480
VP2    AGCCTGCTGCACAGCGTCGGCCAGGACATCTTCAACAGCCTGAGCCCCACAAGCCGCCTG 480
C      AGCCTGCTGCACAGCGTCGGCCAGGACATCTTCAACAGCCTGAGCCCCACAAGCCGCCTG 480
       ************************************************************

B      CAAATCCAGAGCAACTTGGTCAACCTCATCCTGAACTCAAGATGGGTGTTCCAAACCACC 540
VP2    CAAATCCAGAGCAACTTGGTCAACCTCATCCTGAACTCAAGATGGGTGTTCCAAACCACC 540
C      CAAATCCAGAGCAACTTGGTCAACCTCATCCTGAACTCAAGATGGGTGTTCCAAACCACC 540
       ************************************************************

B      GCCAGCCAGAACCAGGGGCTGCTGAGCGGCGAAGCCATCCTGATCCCCGAACACATCGGC 600
VP2    GCCAGCCAGAACCAGGGGCTGCTGAGCGGCGAAGCCATCCTGATCCCCGAACACATCGGC 600
C      GCCAGCCAGAACCAGGGGCTGCTGAGCGGCGAAGCCATCCTGATCCCCGAACACATCGGC 600
       ************************************************************

B      GGCACCCTGCAACAGCAGACACCCGACTGGCTGCTGCCACTGGTGCTGGGGTTGTCCGGC 660
VP2    GGCACCCTGCAACAGCAGACACCCGACTGGCTGCTGCCACTGGTGCTGGGGTTGTCCGGC 660
C      GGCACCCTGCAACAGCAGACACCCGACTGGCTGCTGCCACTGGTGCTGGGGTTGTCCGGC 660
       ************************************************************

B      TACATCAGCCCCGAGCTGCAAGTCATCGAGGACGGGACAAAGAAGAAGTCAATCATTCAT 720
VP2    TACATCAGCCCCGAGCTGCAAGTCATCGAGGACGGGACAAAGAAGAAGTCAATCATTCAT 720
C      TACATCAGCCCCGAGCTGCAAGTCATCGAGGACGGGACAAAGAAGAAGTCAATCATTCAT 720
       ************************************************************

B      CTCTGA 726
VP2    CTCTGA 726
C      CTCTGA 726
       ******
```

Fig. S4: Sequencing results for inserts VP1 (A) and VP2 (B) in recombinant bacmids in baculovirus preparation. PCR method was used for verification (see chapter 4.4.2.). Two sets of primers were used to cover the sequence of VP1 insert. Inserted sequences were identical to template sequences. Alignment was performed in ClusthalW2.